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# PHARMACOGNOSTICAL AND MEDICINAL INVESTIGATION AND APPLICATIONS OF *WITHANIA COAGULANS* (DUNAL) FRUITS

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## ABSTRACT

*Withania coagulans* plays a significant traditional role to treat a large number of human diseases. The present study aimed at determining phytochemical screening, antibacterial, antifungal and antioxidant activities of *W. coagulans*. The study was carried out in Dera Ismail Khan KPK Pakistan in 2017For plant collection, identification; preparation and fractionation of crude extract and preparation of culture media standard procedures were followed. Alkaloid, sugar, and terpanoid were present in all fractions. Flavonoid was present in methanolic and aqueous extract. Saponin was present in ethyl acetate, benzene, n-hexane and chloroform... Disc diffusion method was applied to check antibacterial activities of all the extracts against 5 bacterial strains using Moxifloxacin as reference. Highest antibacterial activity was shown by methanolic fruit extract

against *Proteus vulgaris* giving 15.5mm zone of inhibition. The n-hexane showed minimum antibacterial activity at 500µg/ml concentration giving 4.5mm against *B. subtilis* followed by 5.0mm against *S. aureus*, The aqueous extract gave most conspicuous zone (24.1mm) against *A. niger*. N-hexxane and benzene showed minimum inhibitory activities against all strains. Ethyl acetate and chloroform gave moderate antifungal activities. Antioxidant activity ascended with increasing concentration of all the extract however;.

**Keywords: Antibacterial, Antifunga, Antioxidant Activities Of *Withania, Coagulans* (Dunal) Fruits**

## INRODUCTION

Medicinal plants play a rigorous role in fabricating and designing of novel drugs in the modern era. About 25 – 30% allopathic drugs in the United States are thought to derive one or more constituents from phytomedicinal sources[1-2] *Withania coagulans* also called as Indian rennet in English or Paneerbooti in Urdu and Ning Gu Shui Qie in Chinese is a perennial shrub belonging from family Solanaceae. This plant grows in tropical sub-tropical areas. This plant is found throughout the Pakistan and is routinely cultivated in the rural areas for economical and ethnomedicinal purposes. Besides this, *Withania coagulans* is also found in India, Iran, Nepal, Maldives etc. In Pakistan, most peoples use the fruits of this plant for daily medicinal purposes. Its usage against various abdominal ailments in human and domestic cattle is a general practice in Pakistani rural areas. The *W. coagulans* fruit is considered useful for diabetes [3-4-5] indigestion, liver disorders, dental diseases, mental tension, anorexia, colic, liver cancer, asthma, intestinal problems, elevated blood pressure, menstrual irregularities, insomnia, dyspepsia, flatulence and constipation. It is also used as hepatoprotective, anthelmintic, blood purifier, cytotoxic, anti-inflammatory, antimicrobial, antioxidant, sedative, immunity booster and diuretic[6-7]. It repairs the pancreatic beta-cells which then secret a sufficient amount of insulin which ensures digestion of blood glucose. It also removes toxic matters from the body (*http*). The aqueous extract is orally given to newborns for cleaning their intestines[8-10]. *W. coagulans* is also considered useful in organ transplant especially when the body immune system rejects the new organ [11].

## MATERIALS AND METHODS

### Source of tested microorganism

Pure cultures of five bacterial strains namely *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Klabsiela pneumonia, Bacillus subtilis* and four fungal strains of

*Candida albicans, Penicillium citrinum, Aspergillus niger* and *alternaria* were obtained from microbiology lab of pharmacy department of Gomal University, D. I. Khan, which were then maintained on nutrient broth culture media.

**Plant Collection and identification**

Dried fruits of *W. coagulans* were purchased from the local grocery shop in the month of October, 2017 then identified and authenticated by a senior professor of Govt. Post Graduate College, Bannu. The hulls from the fruits were removed, washed with distilled water to clean any dust or impurities, and then stored at room temperature for 20 days. After thoroughly drying, the fruits were crushed into coarse powder by electrical grinder then stored in clean, sterilized container for further use.

**Preparation of crude extract**

For extraction standard procedure was adapted (Harborne et al., 1973).The shade-dried preserved 793g powder was chopped and soaked in methanol for 15 days, underwent vigorous shaking 7 times every day so as to thoroughly dissolve its material in the methanol. The filtrate was passed through vacuum rotary evaporator under controlled environment i.e. reduced pressure and 40$\overset{o}{C}$ so as to obtain its crude extracts. The extract was further incubated for 48 hours for further exhaustion. The extract was weighed and preserved for onward investigation.

**Fractionation of crude extract**

The crude extract of *W. coagulans* was suspended in water and partitioned sequentially with ethyl acetate, benzene, n-haxane, chloroform and methanol to get their respective fractions. Thus corresponding solvent of each fraction was subjected again to rotary evaporator under controlled environment. Finally, gummy residues of every fraction were obtained.

**Phytochemical screening**

Phytochemical screening of the fractions was conducted following the standard protocols.

**Test for Alkaloid**

Alkaloids were observed using the Dragendroff's Test. Extracts were individually dissolved in diluted HCL and filtered. Each filtrate was treated with Dragendroff's reagent. Dragendroff's reagent is a solution of Potassium bismuth iodide. Formation of red, brown or pinkish purple precipitate indicates the presence of alkaloids.

**Test for Carbohydrates**

A small quantity (0.5gm) of each of the extract was individually dissolved in 5ml of distilled water and filtered. The filtrates

were subjected to Molisch's test which detects the presence of carbohydrates. Moreover the presence of reducing sugar was confirmed by adding Fehling's reagent which produces red brick color.

**Test for Terpenoids**

Salkowski test was followed. A small quantity (0.5g) of each of the extract was mixed with 02ml of chloroform in a test tube to which 3ml conc. $H_2SO_4$ was added to make a layer. Appearance of red-brown color at the interface suggests the presence of terpenoids.

**Test for Flavonoids**

Alkaline reagent test was followed to detect Flavonoids. The extracts were treated with a few drops of NaOH solution, appearance of dark yellow color which becomes colorless if treated with dilute acid. This process indicates the presence of flavonoids.

**Test for Saponin**

Froth Test procedure was adopted. Each of the extract was diluted with 20ml of distilled water in a graduate cylinder and vigorously shaken for 20 minutes; formation of 01 cm thick layer of foam or froth suggests the presence of saponin.

**Test for Tannin**

Gelatin test was followed. To each extract 1% gelatin solution having NaCl was added; formation of whitish precipitates suggests the presence of tannin.

**Table 1: Phytochemical screening of the fruits of *W. coagulans***

| Extract | Alkaloid | Carbohydrate | Terpenoid | Flavonoid | Saponin | Tannin |
|---|---|---|---|---|---|---|
| ethyl acetate | + | + | + | - | + | - |
| benzene | + | + | + | - | + | - |
| n-hexane | + | + | + | - | + | - |
| chloroform | + | + | + | - | + | - |
| methanol | + | + | + | + | - | + |
| Water | + | + | + | + | - | - |
| | + | + | + | + | - | - |

**Preparation of nutrient media**

For cultivation of bacterial culture, the nutrient medium was prepared by suspending 28g agar powder in 01 liter distilled water. Then 0.5%/5g peptone, 0.3%/3g beef extract, and 0.3%/3g NaCl were also added. Heated and stirred the mixture to complete dissolving. Autoclaved it at 121°C for 20 minutes under 15 Ibs pressure adjusting the pH to 7.4 and poured into petri dishes assuring aseptic condition under laminar flow hood and then allowed to solidify for 25 minutes.

**Preparation of stock solution**

Each previously prepared fraction i.e. ethyl acetate, benzene, n-hexane, chloroform

and methanol was dissolved in DMSO (DimethyleSulfoxide) with ratio of 5mg/ml because it did not exhibit any antibacterial and antifungal activity. Furthermore, DMSO also possesses a polar and non-polar group that's why most similar compounds have affinity for it. The crude extracts of the respective plant and their subsequent soluble fractions were then undergone for antibacterial and antifungal activities.

**Antibacterial activity**

In this study, the ethyl acetate, benzene, n-hexane, chloroform and methanolic fractions were tested for antibacterial activities against five bacteria i.e. *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Klabsiela pneumonia, Bacillus subtilis* by using the disc diffusion method i.e. sterile discs (6mm) of Whattman No. 01 paper containing 3 different concentrations, its control of respective solvent and the antibiotic moxifloxacin as standard reference as positive control. These discs were properly air dried at room temperature to shed any residual contents which might interfere with sterilization, determination and inoculation. The said bacteria were separately proceed using sterile swabs over sterilized culture medium plates which were initially placed at low temperature for 2 hours to let maximum diffusion of the said chemicals from the discs into the agar plates then incubated at 37°C. The zones of inhibition were measured after 24 hours. The zones of inhibitions of crude extract, ethyl acetate, benzene, n-hexane, chloroform, methanol and aqueous fraction were compared with the reference drug (Moxifloxacin) then each zone was measured.

**Antifungal activity**

The crude extract, ethyl acetate, benzene, n-hexane, chloroform, methanol and aqueous fraction were subjected to determine (*in vitro*) their antifungal activities against four fungal strains of *Candida albicans, Penicillium citrinum, Aspergillus niger* and *alternaria*. Agar tube dilution method was used following[2, 3]. Sabouraud Dextrose Agar (SDA) was prepared following the manufacturer manual, then 4 ml was dispensed into screw cap tubes which were autoclaved at 121°C for 15 mins, kept in slanting position then allowed to solidify at room temperature. Similarly, the test samples were dissolved in sterile Dimethyl Sulfoxide (DMSO) which functioned as stock solution. The sets of 3 dilutions (5, 10, 15 µg/ml) of *Withania coagulans* fruits extracts/fractions and standard drugs were prepared in double distilled water. Each tube was inoculated with a 4mm diameter piece of inoculum

removed from a 7days old fungal culture. For non mycelial growth, the agar surface streak of *Candida albicans, Penicillium citrinum, Aspergillu sniger* and *alternaria* was applied. Inhibition of the fungal growth was observed after seven days of incubation at 28˚C. Griseofulvin was used as a positive control.

**Antioxidant activity**

Antioxidant activity of the extracts was checked by DPPH assay devised by Blois (1958). Due to paramagnatism conducted by its spare electron, the DPPH is considered a potent radical. In pure ethanol, the solution gives a deep violet color and shows a strong absorption band at 520nm in spectrophotometer. DPPH radical has the ability to accept an electron or hydrogen radical to acquire a stable diamagnetic state thus converting into pale violet color. If a matter to check its antioxidant activity, is mixed with DPPH solution and provide a pale violet color will suggest to have antioxidant activity by free radical scavenging. Lower absorbance value will indicate the higher free radical scavenging activity. Evaluating the antioxidant activity of *Withaniacoagulans*fuit (Dunal), DPPH free radical scavenging assay devised by Blois was used with slight modification.

The crude, Ethyl acetate, methanol, and chloroform extracts were dissolved in absolute ethanol while the aqueous extract in distilled water then each sample was diluted for 5 concentrations two folds. Similarly, 6×10-5 M of DPPH was prepared in absolute ethanol. 500µl of each solution was taken in eppendorf tubes to which 500µl of DPPH was added, vigorously shaken and stood at room temperature for 30 minutes. Trolox was used as positive control. Absorbance was measured at 517nm with 500µl absolute ethanol as blank. DPPH scavenging activity was calculated by the following formula.

**%inhibition = OD control – OD sample × 100/OD control**

**RESULTS**

**Phytochemical screening** (**Table 1** and **figure 1**).

**Antifungal activity**

Antifungal is anything that kills fungi, retard or inhibit their growth or stall reproduction such as nystatin, griseofulvin, fluconazole, amphoterin etc. The crude extract, ethyl acetate, benzene, n-haxane, chloroform, methanol and aqueous fractions were subjected to determine antifungal activities against four fungal strains of *Candida albicans, Penicillium citrinum, Aspergillus niger* and *alternaria* by tube dilution method. The zones of inhibition

obtained were compared with the zones of inhibition of the standard antifungal drug, the Griseofulvin. Most conspicuous inhibition zone (24.1mm) against *A. niger* was obtained from aqueous extract by C3 concentration (15µg/ml) which was followed by methanolic extract (23mm) against the same fungal strain by C3 concentration (15µg/ml) which was followed by methanolic extract giving 22.8 mm zone of inhibition against *P. citrinum*with the same concentration. Ethyl acetate and chloroform exhibited moderate antifungal activities at C3 concentration against *A. niger*, *P. citrinum*, and *C. albicans*. Similarly, n-hexane and benzene showed minimum inhibitory activities against all the fungal strains at all defined concentrations (**Table 2** and **Figure 2**).

**Antioxidant activity**

The phytochemical screening of *Withaniacoagulans* confirmed the presence of alkaloids, carbohydrates, terpenoid, flavonoid, saponin and tannin. All these phyto-constituents are thought to be responsible for antioxidant activities[10]. The fruit extracts of *Withania coagulans* showed good antioxidant activity which may be due to the existence of phenolic compounds in the plant. This free radical scavenging activity is associated with –OH groups found in the chemical structure of the compounds[10]. The *W. coagulans* metabolites like phenolic contents, saponin, tennin, and flavonoid contain antioxidant activities[12] The antioxidant activities of ethyl acetate, methanol, chloroform and aqueous extracts were compared with different concentrations of ascorbic acid. The antioxidant activity ascended with increasing concentrations of the extracts as shown in **table 3** and **figure 3**. Ethyl acetate and aqueous extracts exhibited very good antioxidant activities followed by methanol while chloroform extract showed moderate antioxidant activity.

**Table 1: Antibacterial activity of *W. coagulans***

| Extract/Drug | Concentration µg/ml | Zone of inhibition (mm) | | | | |
|---|---|---|---|---|---|---|
| | | *S. aureus* | *E. coli* | *P. vulgaris* | *K.Pneumonia* | *B. subtilis* |
| Moxifloxacin (used as reference drug) | C1. 25 | 10 | 14 | 14 | 11 | 13 |
| | C2. 50 | 13 | 15 | 15 | 14 | 14 |
| | C3. 100 | 14 | 16 | 15 | 16 | 16 |
| Ethyl Acetate | C1. 25 | 7 | 11 | 8 | 9 | 11.7 |
| | C2. 50 | 8.5 | 13 | 10.3 | 11 | 13 |
| | C3. 100 | 10 | 14 | 12 | 14 | 15 |
| Benzene | C1. 25 | 9 | 7 | 8 | 3 | 7 |
| | C2. 50 | 12 | 10.2 | 9 | 5 | 8 |
| | C3. 100 | 13 | 11.8 | 10 | 10 | 9.5 |
| n-haxan | C1. 25 | 0 | 10 | 2 | 4 | 1 |
| | C2. 50 | 2 | 11 | 5 | 5.5 | 2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | C3. 500 | 5 | 13 | 8 | 7 | 4.5 |
| Chloroform | C1. 25 | 8.5 | 12.9 | 11.2 | 8 | 8 |
| | C2. 50 | 12 | 14 | 13 | 11 | 11 |
| | C3. 100 | 14 | 15.1 | 14 | 13 | 13.5 |
| Methanol | C1. 25 | 9.7 | 13 | 13 | 10.1 | 12.2 |
| | C2. 50 | 13 | 14.7 | 14 | 13 | 13 |
| | C3. 100 | 13.9 | 15.2 | 15.5 | 15 | 15 |
| Aqueous | C1. 25 | 9 | 13 | 13 | 10 | 12.1 |
| | C2. 50 | 12 | 14 | 13.3 | 12.8 | 13 |
| | C3. 100 | 13.3 | 14.7 | 14 | 15.2 | 15 |

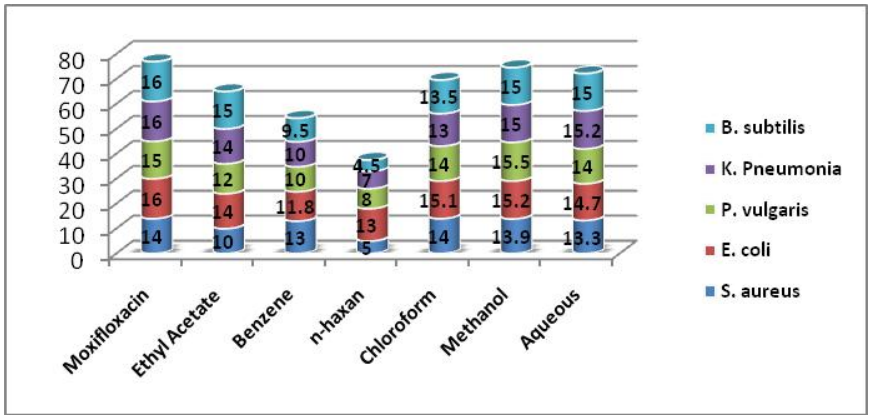


**Figure: 1 Antibacterial activity**

**Table: 2 (Antifungal activity of *W. coagulans*)**

| Extract/Drug | Concentration µg/ml | Zone of inhibition (mm) | | | |
|---|---|---|---|---|---|
| | | *C. albicans* | *P. citrinum* | *A. niger* | *Alternaria* |
| Grisofulvin (used as reference) | C1. 5 | 18 | 18 | 19 | 19 |
| | C2. 10 | 21 | 22 | 23 | 20 |
| | C3. 15 | 22 | 24 | 25 | 24 |
| Ethyl. Acetate | C1. 5 | 16 | 16 | 18 | 11.7 |
| | C2. 10 | 17 | 17 | 19 | 13 |
| | C3. 15 | 18 | 17.8 | 20 | 15 |
| Benzene | C1. 5 | 9 | 11 | 6 | 7 |
| | C2. 10 | 11 | 12.8 | 8 | 8 |
| | C3. 15 | 13 | 13.2 | 10 | 9.5 |
| n-haxan | C1. 5 | 4 | 5.5 | 4 | 1 |
| | C2. 10 | 7 | 8 | 5.5 | 2 |
| | C3. 15 | 8 | 9 | 7 | 4.5 |
| Chloroform | C1. 5 | 9 | 12 | 9 | 8 |
| | C2. 10 | 12 | 14 | 11 | 12 |
| | C3. 15 | 14.8 | 15 | 14 | 13 |
| Methanol | C1. 5 | 17 | 17.3 | 18.2 | 17.1 |
| | C2. 10 | 18 | 21 | 19 | 19 |
| | C3. 15 | 20.9 | 22.8 | 23 | 22 |
| Aqueous | C1. 5 | 18 | 17 | 17.6 | 18 |
| | C2. 10 | 19 | 19.9 | 22 | 19 |
| | C3. 15 | 20 | 22 | 24.1 | 23 |

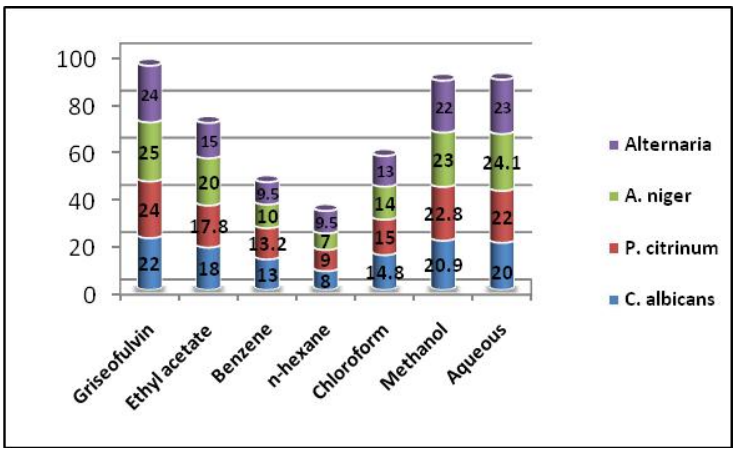


**Figure 2: Antifungal activity**

**Table: 3 (Antioxidant activity of *W. coagulans*)**

| Concentration (µg/ml) | %inhibition | | | | |
|---|---|---|---|---|---|
| | ascorbic acid | ethyl acetate | Methanol | Chloroform | Aqueous |
| 2 | 20.18±0.14 | 25.02±0.10 | 14.10±0.04 | 10.02±0.06 | 18.04±0.04 |
| 4 | 32.18±0.12 | 38.80±0.06 | 27.17±0.04 | 12.16±0.03 | 32.14±0.06 |
| 6 | 48.31±0.14 | 52.06±0.12 | 42.12±0.07 | 14.06±0.03 | 46.66±0.08 |
| 8 | 57.15±0.13 | 62.54±0.08 | 56.16±0.10 | 16.31±0.04 | 58.16±0.13 |
| 10 | 72.56±0.24 | 78.07±0.12 | 67.56±0.14 | 20.22±0.10 | 71.06±0.12 |

## DISCUSSION

Standard protocol was implied to investigate the phyto-constituents in the *Withania coagulans* fruits. Various extracts such as ethyl acetate, benzene, n-hexane, chloroform, methanol and aqueous were made and screened for the occurrence of alkaloids, carbohydrates, terpenoids, flavonoids, saponins and tennin. Ethyl acetate revealed good concentration of alkaloids, sugars, terpenoids and saponin but no flavonoid and tennin. Benzene showed good result for the presence of alkaloid, reducing sugar, terpenoid and saponin but no flavonoid and tennin. n-hexane possessed alkaloid, sugar, terpenoid but no flavonoid, saponin and tennin. Similarly, chloroform had alkaloid, sugar, terpenoid and saponin but no flavonoid and tennin. methanol contained alkaloid, carbohydrates, terpenoid, flavonoid and tennin but no saponin. The aqueous extract showed to have alkaloid, sugar, terpenoid, flavonoid but no saponin and tennin.

### Antibacterial activity

Medicinal plants are widely used as folklore against a wide range of ailments. According to W.H.O medicinal plants or herbal sources are used by 80% of world population.

Antibacterial means the phytochemical property of a phyto-constituent to kill or destroy bacteria or suppress their growth and reproduction ability. Heat, acids, certain chemicals or drugs and antibiotics all have antibacterial characteristics. Antibacterial domains are considered to have negative impact on human health. The result of antibacterial activity of various extracts of the selected plants is shown in table 2. The various fruit extracts of *Withania coagulans* such as ethyl acetate, benzene, n-hexane, chloroform, methanol and aqueous were tested for their antibacterial activity against *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Klabsiela pneumonia, Bacillus subtilis* by using the disc diffusion method compared with Moxifloxacin as standard. As a whole the chloroform, methanol and aqueous extract showed good inhibitory properties. Highest activity was shown by methanolic fruit extract giving 15.5mm zone against *P. vulgaris* followed by 15.2mm against *E. coli*.[11-12] Aqueous fruit extract gave 15.2mm inhibition zone against *K. penumoia* followed by 14.7mm against E. coli. The n-hexane showed minimum activity at C3 concentration (4.5mm) against *B. subtilis* followed by 5mm against *S. aureus*, however; it showed moderate activity i.e. 13mm against *E. coli*. Ethyl acetate also showed moderate inhibitory activity against the different strains at different concentrations.[13]

**CONCLUSION**

*Withina coagulans* is one of the most important medicinal plants folklorically used from remote past to treat a large number of human and cattle diseases and truly considered as a golden remedy for nervous exhaustion, wasting disease, impotence, diabetes, liver complaints etc.[4,7]. Several studies have been conducted to screen its various constituents, and efficacy against various disorders. In the current study highest antibacterial activity was shown by methanolic fruit extract against *Proteous vulgaris* (15.5mm) followed by aqueous extract against *E. coli* (15.2mm) while n-hexane fraction showed minimum activity all bacterial strains. Similarly, most conspicuous antifungal inhibition zone was achieved by aqueous extract against *Aspergilis niger* (24.1mm) followed by methanolic extract against *A. niger* (23mm) while n–hexane extract showed minimum inhibitory activity against all the fungal strains. *Withania coagulans* contained saponin, tannin, flavonoids etc. Ethyl acetate and aqueous extract showed very good antioxidant activity which may be due to the phenolic contents, alkaloids, carbohydrates, terpenoid,

flavonoid, saponin and tannin. The current study is not the last goal but it needs more *in vitro* and *in vivo* confirmation.

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