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# IN VITRO ANTI-MICROBIAL AND CYTOTOXIC ACTIVITY ANALYSIS OF CISSUS ADNATA IN DIFFERENT FRACTIONS

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## ABSTRACT

This study provides a scientific basis for the use of *Cissus adnata* in traditional medicine. The whole plant was extracted using methanol and different partitionates were obtained using different organic solvents, like, dichloromethane, petroleum ether, and chloroform. All these fractions were subjected to cytotoxic activity and antimicrobial activity determination. Among all the partionates dichloromethane fraction provides the highest $LC_{50}$ value of 6.79 µg / ml. After evaluating the antimicrobial activity of *Cissus adnata* the methanolic extract show highest effect on different Gram positive bacteria, Gram negative bacteria and different fungal strain as well where Kanamycin was used as standard. Among Gram positive bacterial strains *Bacillus subtilis*, from Gram negative bacterial strains *E. coli*, *Vibrio mimicus*, *Vibrio parahemolyticus* and from Fungal strains *Sacharomyces cerevacae* was inhibited greatly.

**KEYWORDS:** Antimicrobial, Cytotoxicity, *Cissus adnata*, Disc diffusion assay**,** Brine shrimp lethality bioassay.

## INTRODUCTION

Interest in medicinal plants increasing day by day because they are relatively safer than most modern medicines. In addition, they are more economical and ecological for local conditions. The use of natural products and their greater durability is a clear advantage over some countries. For Bangladesh medicinal plants and compounds which derived from them are an essential part of the health system. Moreover, plant derivative has a large market for raw medicinal plants as dietary supplements, and for therapeutic purposes in developed and developing countries around the world.[1]

Cissus adnata is a medicinal plant belongs to Vitaceae,[8,9] Common name of this plant is Grape, Heart-leaved. Cissus adnata is a plant which stem size is around 9 cm in diameters. Leaf size is about 6.5-19 x 5.5-19 cm and petioles is about 2.5 to 12 cm long. Flowers are 3mm in diameter and inflorescence leaf opposed. Fruits become black when they ripe. Seeds are 22-24 x 25 mm and they are found in the monsoon forest, in the beach forest and the located more in tropical forests. It also available in Asia and Malaysia.

Other plants of Vitaceae family also have various types of medicinal properties. Ampelocissus latifolia is a plant of Vitaceae family showed antimicrobial and antioxidant potential.[14] Moreover, this plant's fruit showed analgesic and anti-inflammatory activities.[13] Cayratia trifolia plant has the antiprotozoal, hypoglycemic, anticancer, diuretic antiviral and antibacterial activity.[15] Vitis rotundifolia plant has Antioxidant, Antibacterial, and Antibiofilm Properties.[16] .The compounds which are found in plant has various pharmacological activities like antimicrobial.[7,10] And now a days bacteria are becoming resistance to available medicine so new compound need to introduce to overcome this situation.[11] Moreover, anticancer drugs which are derived from plant source have good level of potential and recent drugs which are currently serving as anticancer drug most of them are natural derived.[12] Nearly 87% of crucial medicine such as anticancer, antibiotic come from natural products and 28% natural chemicals come from nature.[3]

In addition, 21,000 species have great pharmacological effects which can be used as medicinal plants and able to serve medicine world with different types of therapeutically active compound. Organizations like ESCOP (European Organization Cooperative On Phototherapy, 1999), German Commission E and WHO (World Health Organization) stated that to cure diseases use of plants increases gradually every day. For this reason this research was focused on to identify the antimicrobial activity by disc diffusion assay and the cytotoxic activity by brine shrimp lethality bioassay.

## MATERIALS AND METHODS

### Plant collection

The whole plant was collected from Bikrampur in the district of Munshiginj in February, 2017. After the collection it was identified by the National Herbarium of Bangladesh in Mirpur, Dhaka. The identification number was 45961. Our plant sample was given to the herbarium authority for preservation.

**Preparation of plant extract**

After proper washing the whole plant was shade dried under the sun for a few week. The dried plant was ground to a coarse powder and extracted by soaking it in 2.5 liters of methanol. The mixture was kept for 15 days and occasional stirring was maintained. The entire mixture was filtered through a perfect cotton plug and finally with No. 1 Watmann filter paper. The filtrate volume was reduced by using a rotary evaporator at a low temperature as the compounds can be degraded at high temperature. The total weight of the dried extract was 40g.

**Chemicals and Drugs**

Vincristine sulphate was obtained from Beacon Pharmaceuticals Limited, Bangladesh, Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific, UK. Methanol, Petroleum ether, Dichloromethane, Chloroform acquired from Active Fine Chemicals Limited., Bangladesh. Furthermore, Nutrient Agar, Nutrient Broth, M. H. Agar was purchased from Becton Dickinson GmbH, Germany and Kanamycin was obtained from Beximco Pharmaceuticals Limited, Bangladesh.

**Partitioning of plant extract**

2 to 3 g of sample were taken in conical flasks separated by 100 ml and 50 ml. Solvents such as methanol, chloroform, petroleum ether, dichloromethane and water added separately. All the flasks were labeled, capped with cotton stoppers and allowed to stand for 1 to 2 hours and filtered using No.1 Watmann filter paper.[17,18,19]

**Brine shrimp lethality bioassay**

This bioassay reveals cytotoxicity and in addition a wide variety of pharmacological exercises, for example, antimicrobials, antivirals, pesticides and tumor-resistant, etc. mixtures.[2,4] 38 g of sea salt (NaCl) were weighed, dissolved in one liter water and filtered to obtain a clear solution. *Artemia salina* was taken from the small tank that contained salt water and incubated the shrimp and developed as nauplii. Tank provided with a constant supply of oxygen and a table lamp was used for lighting. Using a Pasteur pipette, 10 live shrimps were added to each of the test tubes containing 5 ml of seawater. All test substances were removed from vials and comminuted in 100 µl of dimethylsulfoxide Non-adulterated (DMSO) to obtain stock preparations. At this point, 50 µl of solution was taken from the main test tube containing 5 ml of seawater and 10 nauplii. In this way, the last concentration was prepared in the primary test tube was 400 µg / ml. At this stage, a progression of the preparations of

different approaches has been established from the preparation of the stock through a strategy of series dilation method. For each condition, 50 µl of test sample were added to the test tube and 50 µl of new DMSO were added to the vial which results separate approaches have been found in the different test tubes.

**Table 1: Test samples with concentration values after serial dilution.**

| Test Tube No. | Concentration (µg/ml) |
|---|---|
| 1 | 400 |
| 2 | 200 |
| 3 | 100 |
| 4 | 50 |
| 5 | 25 |
| 6 | 12.5 |
| 7 | 6.25 |
| 8 | 3.125 |
| 9 | 1.5625 |
| 10 | 0.78125 |

**Disc diffusion assay**

In this established methods, anti-microbial diffuse from a restricted source through the supplement agar gel and make a microbial inhibition. Dried and cleaned filter paper circles (6 mm breadth) containing the test samples of known concentration are set on supplemented agar medium consistently inoculated with the test microorganisms. Standard anti-microbial (kanamycin) plates and clear circles are utilized as positive and negative control. These plates were kept at low temperature (4°C) for 24 hours to permit greatest dispersion of the test materials to the encompassing media.[5] The plates were then rearranged and hatched at 37°C for 24 hours for ideal development of the creatures. The test materials having antimicrobial property restrain microbial development in the media encompassing the plates and consequently yield a unique, particular range characterized as zone of inhibition. The antimicrobial movement of the test sample was then dictated by measuring the distance across of zone of inhibition measured in millimeter.[5,6] In the present study the unrefined samples and also divisions were tested for antimicrobial action. The examination was done once and the reading was analyzed.[6]

**Collection of bacterial strains**

Different gram positive and gram negative bacterial and fungal stains were collected from the microbiology lab of State University of Bangladesh and Mathematics & Natural Science Department of BRAC University.

**Table 2: Different strains used in antimicrobial screening.**

| Gram positive Bacteria | Gram negative Bacteria | Fungi |
|---|---|---|
| | *Escherichia coli* | |
| *Bacillus cereus* | *Salmonella paratyphi Salmonella typhi* | |
| *Bacillus megaterium* | *Shigella boydii* | *Aspergillus niger* |
| *Bacillus subtilis* | *Shigella dysenteriae* | *Candida albicans* |
| *Sarcina lutea* | *Pseudomonas aeruginosa* | *Sacharomyces cerevacae* |
| *Staphylococcus aureus* | *Vibrio mimicus* | |
| | *Vibrio parahemolyticus* | |

**Sterilization procedure**

To prevent any kind of cross contamination by the test living beings the antimicrobial screening was done in Laminar Hood and a wide range of precautionary measures were exceptionally kept. UV light was used as microbial killer before start working in the Laminar Hood. Petridishes and other dishes were cleaned via autoclaving at a temperature of $121^0$C and a weight of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, clear plates and so forth were additionally cleaned by UV light.

**Preparation of subculture**

In an aseptic condition under laminar airflow cabinet, the test microorganisms were inoculated in the nutrient agar plates from the stock culture. The immunized strains were then incubated for 24 hours at $37^0$C for their ideal development. The isolated colonies were used for antimicrobial screening purpose.

**Preparation of the test plate**

The test organisms were exchanged from the subculture to the test tubes containing around 10 ml of liquefied and disinfected nutrient broth medium with the assistance of a sanitized loop circle in an aseptic region. The test tubes had been shaken by vortex to get a uniform suspension of the test microorganism. The bacterial suspension was transferred to the sanitized petridishes by using cotton swaps.

**Preparation of discs**

Test sample was measured and dissolved in specific amount of DMSO to get the desired concentration under an aseptic condition. Disinfected cuttings of filter paper were taken in a clear petridish under the laminar hood. At this point, the circles were soaked in test samples and dried.

**Table 3: Preparation of sample Discs.**

| Plant part | Test Sample | Dose (µg/disc) | Required amount for 20 disc (mg) |
|---|---|---|---|
| Whole plant of *Cissus Adnata* | Methanolic extract | 400 | 8.0 |
| | Petroleum ether partitionate | 400 | 8.0 |
| | Dichloromethane soluble partitionate | 400 | 8.0 |
| | Chloroform soluble partitionate | 400 | 8.0 |
| | Aqueous soluble partitionate | 400 | 8.0 |

Standard Kanamycin (30 µg/circle) plates were used as positive control and untreated circles were utilized as negative controls.

**Diffusion and incubation**

The treated filter paper's circles, the standard anti-microbial discs and the control circles were set firmly on the M. H. agar plates. The agar plates were pre-inoculated with test microorganisms. The plates were then kept in a cool place at $4^0$C for around 24 hour's to permit adequate dissemination of the materials from the circles to the encompassing agar medium. The plates were then rearranged and kept in an incubator at $37^0$C for 24 hours.

**Determination of the zone of inhibition**

The antimicrobial potency of the test samples were measured by their activity to prevent the growth of the microorganisms surrounding the discs which gave clear zone of inhibition after incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zone of inhibition in millimeter with a transparent scale.

## RESULTS AND DISCUSSION

**Brine shrimp lethality bioassay**

The lethal concentration ($LC_{50}$) represents standard measure of the toxicity of the substance that kill half of the test nauplii in a specific time. In this study vincristine sulphate used as standard showed $LC_{50}$ value was 0.45µg/ml. Compared to the standard among the extractive PESF showed the most potent lethality with value of 1.87µg/ml. The $LC_{50}$ values of ME, PETSF, CSF, and AQSF were found to be 4.4µg/ml, 1.87µg/ml, 3.69 and 4.33µg/ml, respectively (Table 4). After this analysis it was cleared that the plant *Cissus adnata* has cytotoxic activity. Graphical representation showed relationship between percentages of morality and different concentration samples where percentage of mortality increasing with concentration. (Figure 2). Each samples regression line provided in the table 4. So, this plant can used as cytotoxic agent with proper purification and isolation. For other pharmacological activities cytotoxicity should be taken in account.

**Table 4: $LC_{50}$ values of the test samples of whole plants of *Cissus adnata.***

| Test samples | Regression line | $R^2$ | $LC_{50}$ (µg/ml) |
|---|---|---|---|
| VS | y = 30.799x + 60.653 | 0.973 | 0.45 |
| ME | y = 7.4545x +17.091 | 0.8533 | 4.4 |
| PESF | y =6.8182x + 37.273 | 0.6114 | 1.87 |
| DCMSF | y = 11.455x – 27.818 | 0.9439 | 6.79 |
| CSF | y =9.8182x +13.818 | 0.8022 | 3.69 |
| AQSF | y = 8.7273x + 12.182 | 0.8845 | 4.33 |

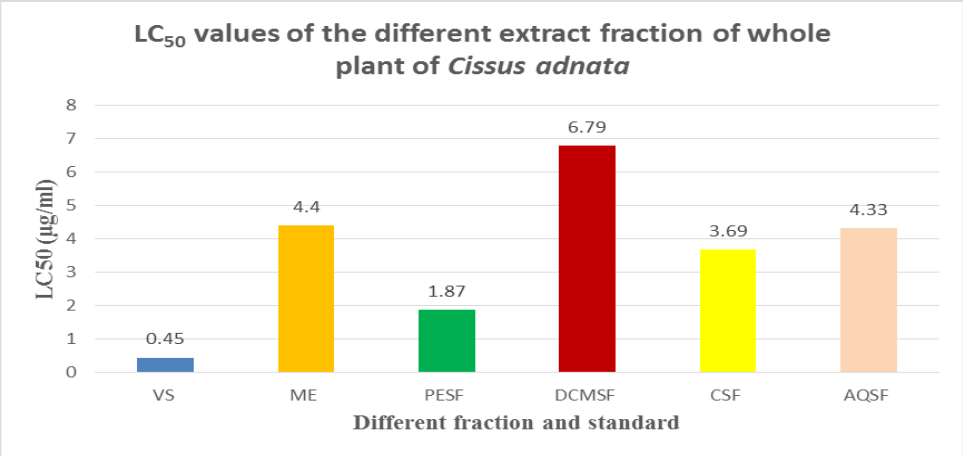


**Figure 1: $LC_{50}$ values of the different extract fraction of whole plant of *Cissus adnata.***

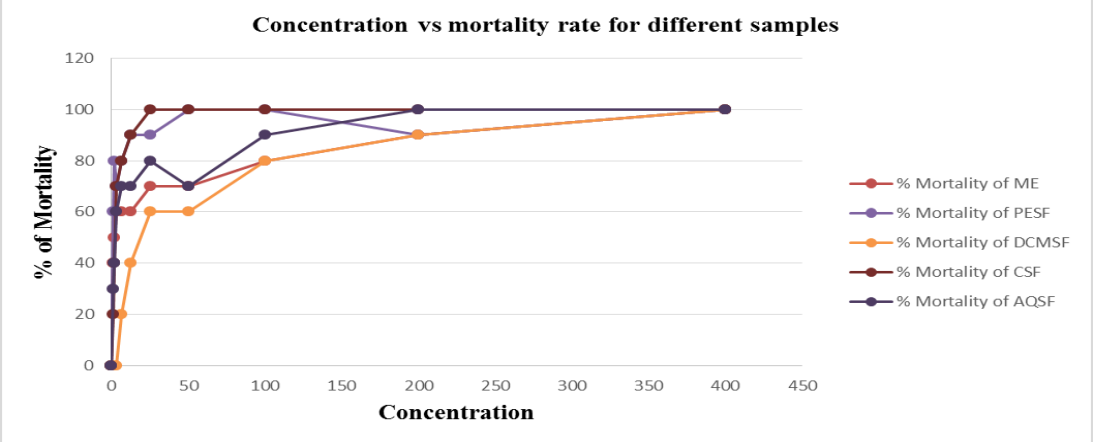


**Figure 2: Graphical representation of % of mortality and different concentration of samples.**

### In vitro antimicrobial screening

The methanolic extract (ME) of whole plant of *Cissus adnata* and its different partitionates i.e. Petroleum ether (PESF), dichloromethane(DCMSF), chloroform (CSF) and aqueous fraction (AQSF) dissolvable portions of whole plant of *Cissus adnata* were subjected to antimicrobial screening with a concentration of 400 µg/ml for each state. Among the extractives, ME revealed highest antimicrobial activity. PESF, DCMSF, CSF and AQSF

showed minimal activity against various microorganisms. The results are given in the table 5. The methalonic fraction sample showed the most outstanding inhibiting activity against microbial growth and having zone of inhibition ranging from 35.0 mm to 40.0 mm. The bigger zone of inhibition observed by ME was 40.0 mm against *Escherichia coli*, *Vibrio mimicus*, *Vibrio parahemolyticus*, *and Sacharomyces cerevacae*. This partitionate additionally indicated direct antifungal activity against *Candida albicans* (having zone of inhibition of 37mm) and *Sacharomyces cerevacae* (having zone of inhibition of 40 mm) and antibacterial action against *S. aureus* (35.0 mm), *S. lutea* (35.0 mm), *S. typhi* (37.0 mm), *V. parahemolyticus* (40.0 mm), *B. cereus* (36.0 mm), *B. megaterium* (36.0 mm), *E. coli* (40.0 mm), *Bacillus subtilis* (38mm), *Pseudomonas aeruginosa* (35mm), *Salmonella paratyphi* (36mm), *Shigella boydii* (36mm) *Shigella dysenteriae* (37mm) (Table 5).

So only ME shows the significant zone of inhibition against microbial growth. Other than that no other solvent extract shows the minimum zone of inhibition against microbial growth.

**Table 5: Antimicrobial activity of test samples of bark of *Cissus adnata.***

| Test microorganisms | Diameter of zone of inhibition (mm) | | | | | |
|---|---|---|---|---|---|---|
| | ME | PESF | DCMSF | CSF | AQSF | Kanamycin |
| **Concentration µg/ml** | 400 | 400 | 400 | 400 | 400 | 200 |
| **Gram positive bacteria** | | | | | | |
| *Bacillus cereus* | 36 | 0 | - | - | - | 37.6 |
| *Bacillus megaterium* | 36 | - | - | -- | - | 38.3 |
| *Bacillus subtilis* | 38 | - | - | 7 | - | 35.0 |
| *Staphylococcus aureus* | 35 | - | - | - | - | 35.0 |
| *Sarcina lutea* | 35 | - | - | - | - | 37.3 |
| **Gram negative bacteria** | | | | | | |
| *Escherichia coli* | 40 | - | 10 | - | - | 37.0 |
| *Pseudomonas aeruginosa* | 35 | - | - | - | - | 35.6 |
| *Salmonella paratyphi* | 36 | - | - | - | - | 35.1 |
| *Salmonella typhi* | 37 | - | 13 | 12 | - | 37.3 |
| *Shigella boydii* | 36 | - | - | 7 | - | 38.0 |
| *Shigella dysenteriae* | 37 | - | - | - | - | 35.6 |
| *Vibrio mimicus* | 40 | - | - | - | - | 36.0 |
| *Vibrio parahemolyticus* | 40 | - | - | - | - | 37.1 |
| **Fungi** | | | | | | |
| *Candida albicans* | 37 | - | - | - | - | 38.3 |
| *Aspergillus niger* | 35 | - | - | - | - | 37.0 |
| *Sacharomyces cerevacae* | 40 | - | - | - | - | 38.6 |

Other than that DCMSF and CSF of *Cissus adnata* showed 13 mm and 12 mm inhibition zone against *Salmonella typhi*. And also against *Escherichia coli* DCMSF gave 10 mm zone

of inhibition. CSF showed 7 mm of inhibition zone for both *Bacillus subtilis* and *Shigella boydii*.

## CONCLUSION

The conduction of Brine Shrimp Lethality Assay denoted there was significant cytotoxic activity in the plant extract of *Cissus adnata*. The petroleum ether fraction showed the most potent result among the other fractionates. The antimicrobial screening indicated moderate level antimicrobial activity. The methanol fraction showed the highest zone of inhibition comparing to the other fractions. Further study can be conducted to purify and isolate the compounds from these fractions which are responsible for activities like cytotoxicity and antibacterial.

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