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# IN VITRO CYTOTOXIC ANALYSIS OF *ADHATODA VASICA* USING *ARTEMIA SALINA* AND *SACCHAROMYCES CEREVISIAE*

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## ABSTRACT

**Background:** Ancient Unani, Egyptian, Chinese and many other manuscripts hold records of healing rituals and therapeutic uses of different herbs, inferring that they were used for medicinal purposes since the first human civilizations. A medicinal herb produces compounds (phytochemicals) which stimulate therapeutic responses and is hence a potential component a medicinal drug formulation. *Adhatoda vasica* a well-known herb of the Acanthaceae family has been frequently used in the Unani and Ayurvedic traditional practices of medicine. Several of its parts (leaves, bark, root, and flowers) are used to treat a multitude of medicinal conditions and several microbial infections.

**Objectives:** An analysis of the phytochemical profile of Vasaka leaves and to study their cytotoxic effect on *Artemia salina* and *Saccharomyces cerevisiae* cytotoxic assays.

**Methods:** Phytochemical profiles for their presence in the methanol extract, chloroform extract and petroleum ether extract of *Adhatoda* were analysed by a number of tests. Cytotoxic effect of the extracts was studied on brine shrimp (%mortality of nauplii) and yeast (%cell death)

**Results:** The phytochemical analysis of the plant extract showed a profile with lot of variations, having a unanimous negative result for proteins and glycosides inferring no presence in any extract. Methanol extract displayed highest toxicity towards brine shrimp, and chloroform extract was the most toxic towards yeast culture.

**Conclusion:** Phytochemical profiles reveal the presence of important bioactive chemicals in plants. The different extracts showed a variation in the presence of different phytochemicals. Furthermore, the

cytotoxic assays of yeast and brine shrimp are convenient, affordable, and readily available to analyse and study the potential of compounds against parasitic and fungal infections.

**Keywords: *Adhatoda vasica*, Brine shrimp lethal assay, Cytotoxicity studies, Phytochemical analysis, Yeast cytotoxic assay**

## INTRODUCTION:

The use of plants for medicinal and therapeutic purposes dates back to our early ancestors of different civilizations. Several manuscripts and old testaments (such as the Vedas and the Bible) hold records of various medicinal plants and their therapeutic uses **[1-3]**. Initially, the medicinal plants were used in their raw forms as various herbal preparations of teas, tinctures, powders, and dressings. The same concoctions were passed down generations of healers for treating various ailments **[4]**. Modern history dating back to the beginning of the 19$^{th}$ century entails the use of medicinal herbs as chemicals or substances that have been isolated from them leading to the field of drug discovery and development. Several drugs with medicinal properties that were used frequently were isolated from these medicinal properties (include drugs such as morphine, codeine, quinine etc.). Furthermore, till current times the various chemicals and substances present in medicinal plants are still being analyzed. Application of drug discovery is evidently used at present to regulate herbal formulations and to identify biological markers for medical conditions **[5-7]**.

Plants frequently face a lot of stresses and toxic compounds as compared to man. They face constant threats from animals, insects, and pathogens, based on which they have efficiently evolved to produce compounds that protect them. These compounds which are secondary metabolites are produced by the various plant biological cycles and are widely known as phytochemicals **[8]**. Several classes of phytochemicals have been identified on the basis of variations in their chemical conformation and structure – alkaloids, flavonoids, saponins, tannins, terpenoids, phenolics, polysterols, essential oils, protease inhibitors, and carotenoids **[9-10]**. Phytochemicals display an array of medicinal benefits such as antimicrobial activity, anti-inflammatory activity, anti-neoplastic activity, anti-genotoxic activity, anti-mutagenic activity, anti-oxidizing activity. Several medicinal plants have been utilized in the day to day living to deal with a variety of medical ailments. Majority of the drugs developed in the clinical sector are based on these natural herbs, and at present the same play a vital role in the development of pharmaceutical drugs. Allopathic medicine makes use of plants extracts made with alcoholic and aqueous solvents that

have been found to show potent antiviral, antimicrobial, and anti-tumor activity **[11]**.

Secondary metabolites produced by plants and microorganisms are used to make a large number of drugs and bioactive compounds of importance. The 19th century documented the pioneer experiments on the antimicrobial characteristics of different components of plants. There are records which state the use of different segments (such as leaves, fruits, flowers, roots, and seeds) of the medicinal herbs for curing particular diseases **[12]**. Among the commonly used medicinal plants is the herb *Adhatoda vasica*, commonly also known as the Malabar nut has been used for over 2 millennium as a therapeutic agent by the Unani and Ayurvedic traditional medicinal systems. It is an endemic plant of Asia, and mostly has been used as an anti-tussive and bronchodilator to treat respiratory distress, lower the blood pressure, and to stimulate anti-inflammation **[13]**. Phytochemical studies of different aerial extracts of the same have confirmed the presence of vasicine, adhatodine, vasicinolone, vasicine acetate, vasicinone, vasicoline, and deoxyvasicinone **[14-15]**. A lot of analyses have been done on the anti-inflammatory and anti-bacterial properties of vasika **[16-18]**, in contrast there are fewer studies on the anti-helminthic and anti-parasitic effects of the same.

The study's purpose is to make a qualitative analysis of the phytochemicals present in *Adhathoda vasica* using different solvents. The plants are commended to treat a spectrum of medical conditions of respiratory distress, asthma, jaundice, gonorrhoea, fever etc. The roots and leaves treat arthritis, dysentery, and diarrhoea effectively, furthermore the leaves show anti-inflammatory and pain-relieving activity for skin disorders.

In the present study, *Adhatoda vasica* has been chosen to carry out cytotoxic studies to show the potential of the herb to treat parasitic and fungal infections and disorders. The study is based on the *in vitro* activity of the herb for different solvent extracts on *Artemia salina* (brine shrimp) and *Saccharomyces cerevisiae*.

## MATERIALS AND METHODS:

### Chemicals and Reagents:

***Solvents for extraction*:** Methanol, Petroleum ether, Chloroform

***Phytochemical analysis*:** Mayer's Reagent, Wagner's Reagent, Concentrated sulphuric acid (conc. $H_2SO_4$), Benedict's reagent, Potassium hydroxide (KOH), Phenolphthalein, Concentrated Hydrochloric Acid (conc. HCl), Chloroform, Ammonia solution, Pyridine, Sodium Nitroprusside (SNP), Sodium Hydroxide (NaOH), Ferric Chloride ($FeCl_3$), Lead Acetate, Acetic anhydride, Millon's reagent, Copper Sulphate Solution ($Cu_2SO_4$), Magnesium pieces.

***Brine shrimp lethality assay*:** NaOH, Sodium Chloride (NaCl), Dimethylsulfoxide (DMSO), Potassium dichromate.

***Yeast cytotoxic assay*:** Potato dextrose broth, 5-Fluorouracil, Methylene blue dye.

**Collection of plant sample:**

Fresh leaves of *Adhatoda vasica* were gathered from the botanical garden of Lokmangal College of Agricultural Biotechnology, Wadala. The leaves were washed using 1% Sodium chloride (NaCl) and dried in the shade for a duration of a week. Following this the dried leaves were powdered with the aid of a mixer.

**Collection of test organisms:**

The dried cysts of *Artemia salina* (eggs of brine shrimp) are collected from the local aquarium shop. The cultures of *Saccharomyces cerevisiae* were collected from the Department of Microbiology and inoculated on sterilized potato dextrose broth at 37°C for a day (seeded broth).

**Extraction:**

The extracts of the plant powder were made by using cold solvents methanol, petroleum ether, and chloroform. The mixtures were placed on a rotatory shaker for a period of 3 days at the temperature condition of 37°C **[19].** The mixtures were then put for solvent evaporation and the extracts were obtained.

**Phytochemical analysis [20]:**

1. **Test for alkaloids:**

**(a) Mayer's test:** To a few ml of the plant sample extract, two drops of Mayer's reagent was added along the sides of the test tube. Appearance of white creamy precipitate indicates the presence of alkaloids.

**(b) Wagner's test:** A few drops of Wagner's reagent were added to few ml of plant extract along the sides of test tube. A reddish-brown precipitate confirms the test as positive.

**2. Test for carbohydrates:**

**(a) Molish's test:** To 2 ml of plant sample extract, two drops of alcoholic solution of α- naphthol were added. The mixture was shaken well and few drops of concentrated sulphuric acid was added slowly along the sides of test tube. A violet ring indicates the presence of carbohydrates.

**(b) Benedict's test:** To 0.5 ml of extract, 0.5 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 minutes. A characteristic coloured precipitate indicates the presence of sugar.

**3. Test for fixed oil and fats:**

**(a) Spot test:** A small quantity of extract was pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oils.

**(b) Saponification test:** A few drops of 0.5 N alcoholic potassium hydroxide solution were added to a small quantity of extract along with a drop of

phenolphthalein. The mixture was heated on a water bath for 2 hours. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

**4. Test for glycosides:**

50 mg of the powdered extract was hydrolysed using concentrated hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate was subjected to the following tests.

**(a) Borntrager's test:** To 2 ml of filtered hydrolysate, 3 ml of chloroform was added and shaken, chloroform layer was separated and 10% ammonia solution was added to it. Pink colour indicates presence of glycosides.

**(b) Legal's test:** 50 mg of extract was dissolved in pyridine, sodium nitroprusside solution was added and made alkaline using 10% NaOH. Presence of glycoside is indicated by pink colour.

**5. Test for phenolic compounds and tannins:**

**(a) Ferric chloride test:** The extract (50 mg) was dissolved in 5 ml of distilled water. To this few drops of neutral 5% ferric chloride solution were added. A dark green colour indicates the presence of phenolic compound.

**(b) Lead acetate test:** The extract (50 mg) was dissolved in of distilled water and to this 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicates the presence of phenolic compounds.

**6. Test for steroids:**

**(a) Libermann-Buchard's test:** The extract (50 mg) was dissolved in of 2 ml acetic anhydride. To this, 1 or 2 drops of concentrated sulphuric acid were added slowly along the sides of the test tube. An array of colour change shows the presence of phytosterols.

**(b) Salkowski test:** 2 mg of dry extract was shaken with chloroform, to the chloroform layer sulphuric acid was added slowly by the sides of test tube. Formation of red colour indicates the presence of steroids.

**7. Test for proteins:**

The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through Whatmann No.1 filter paper and the filtrate was subjected to test for proteins.

**(a) Millon's test:** To 2 ml of filtrate few drops of Millon's reagent were added. A white precipitate indicates the presence of proteins.

**(b) Biuret test:** 2 ml of filtrate was treated with 1 drop of 2% copper sulphate solution. To this 1 ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets. Pink colour ethanolic layer indicates the presence of protein.

**8. Test for flavonoids:**

**(a) Shinoda's test:** 2 mg of extract was dissolved in 5 ml of ethanol and to this 10 drops of dilute hydrochloric acid followed by a small piece of magnesium were added. Formation of pink, reddish or brown colour indicates the presence of flavonoids.

**(b) Sulphuric acid test:** To a few drops of the extract, a few drops of concentrated $H_2SO_4$ was added and shaken well and allowed the content to stand for few minutes. The yellow colour shows the presence of flavonoids.

**In vitro cytotoxic study using *Artemia salina*:**

***Preparation of simulated sea water*:**

7.6 grams sea salt was weighted accurately, dissolved in 200 ml of sterilized distilled water and then filtered to get clear solution. The pH of the sea water was maintained between 8-8.5 using 1N NaOH solution.

***Hatching the brine shrimp eggs*:**

The brine shrimp eggs were hatched in artificial sea water (3.8% NaCl solution) with robust aeration for 48 h day/dark cycles to mature shrimp referred to as nauplii. Constant oxygen supply was performed during the hatching time by using air pump. The hatched eggs were attracted to the lamp on the bottom of tank **[20]**. The cytotoxicity assay was performed on brine shrimp nauplii by using Meyer's method.

***Preparation of sample*:**

For the Cytotoxicity test, 100 mg of each petroleum ether, chloroform and methanol extracts were separately diluted with 1 ml DMSO. In this study, the following groups for treatment were prepared

*Group 1*: Water (1ml) served as negative control

*Group 2*: Potassium dichromate (10 mg/ml) served as positive control

Group 3: Petroleum ether extract at dose of 100 mg/ml

Group 4: Chloroform extract at dose of 100 mg/ml

Group 5: Methanol extract at dose of 100 mg/ml

In each group mentioned above, 3ml of sea water and 10 nauplii were applied.

***Treatment of Sample with brine shrimp nauplii*:**

By making use of the Pasteur pipette 10 living nauplii were added to every one of the vials containing 3 ml of simulated sea water. A magnification glass was used for convenient count of nauplii.

***Counting the nauplii*:**

After one day, the vials were observed using a magnifying glass and the quantity of survival nauplii in each vial were counted and recorded. Because of this data, the percentage of mortality of nauplii was calculated for each and every concentration of the sample.

**In vitro cytotoxic study using *Saccharomyces cerevisiae* [21]:**

***Dilution of seeded broth*:**

The seeded broth was diluted with sterilized distilled water in order to get $25.4\times10^4$ cells/ ml (average).

***Sample preparation*:**

In this study, the following groups for treatment were prepared:

*Group 1*: Water (1ml) served as negative control.

*Group 2*: 5-Flourouracil (10 mg/ml) served as positive control.

*Group 3*: Petroleum ether extract at a low dose of 100 mg/ml.

*Group 4*: Petroleum ether extract at a high dose of 200 mg/ml.

Group 5: Chloroform extract at a low dose of 100 mg/ml.

*Group 6*: Chloroform extract at a high dose of 200 mg/ml.

*Group 7*: Methanol extract at a low dose of 100 mg/ml.

*Group 8*: Methanol extract at a high dose of 200 mg/ml.

***Treatment with yeast cultures*:**

For cell viability count, solution containing 2.5 ml of potato dextrose broth and 0.5 ml of yeast inoculums and following test samples were added in eight separate test tubes and incubated at $37^0$c.

***Cell death count*:**

Next day, the cell death count was done using methylene blue differential staining. In the cell suspension, 0.1% methylene blue dye was added in all tubes and was observed under low power microscope. Viable cells remained unstained while dead cells were stained blue in color. The no. of viable cells was counted in 16 chambers of hemocytometer and the average no. of cells was calculated. Percentage of cell death was calculated.

**RESULTS AND DISCUSSION:**

**Extraction yield:**

Methanol produced the highest extraction yield of 22.5%, followed by chloroform yielding 16.25%, and finally petroleum ether giving a yield of 15% from the plant sample.

Among the three solvents used for solvent extraction, the most yield was produced by methanol **(Figure 1)** which is in accordance with other studies as methanol being a good solvent for extraction as it has a higher power to penetrate the cell into the content of the cells **[22].**

**Phytochemical analysis:**

There was a lot of variance in the qualitative phytochemical analysis of the different solvent extracts of *Adhatoda vasica*. All three solvents showed a unanimous negative result for the presence of glycosides and proteins, confirming an absence of the same **(Table 2)**. Further, a quantitative analysis of the phytochemicals by High Performance Lipid Chromatography (HPLC) must be performed on the sample to confirm the quantity and presence of the different groups of phytochemicals **[23]**.

***In vitro* cytotoxic study using *Artemia salina*:**

The cytotoxic activity of leaves of *Adhatoda vasica* were tested against *Artemia salina* (Brine shrimp eggs).

The number of viable and dead nauplii were counted. The percentage of mortality of nauplii were calculated by using the following formula:

$$\% \text{ Mortality} = \frac{\text{Total number of dead nauplii} * 10}{\text{Total number of nauplii}}$$

**The total number of nauplii for each of the samples was 10**

For the *in vitro* brine shrimp lethality assay, the methanol extract showed the highest %mortality rate, and the cytotoxicity showed a trend of increase as follows: Negative control > AVCE > AVPE > AVME > Positive control

***In vitro* cytotoxic study using *Saccharomyces cerevisiae*:**

The cytotoxic activity of leaves of *Adhatoda vasica* were tested against *Saccharomyces cerevisiae.*

The no. of viable and dead cells were counted in 16 chambers of hemocytometer and percentage of cell death were calculated by following formula:

$$\% \text{ Cell Death} = \frac{\text{Total number of dead cells} * 100}{\text{Total number of cells}}$$

**Table 1: Weight of sample and volume of solvent used for extraction**

| Sr. no. | Weight of powder (kg) | Solvents used | Volume of solvents (L) | Sample: Solvent ratio |
|---|---|---|---|---|
| 1. | 0.08 | Petroleum ether | 0.56 | 1:7 |
| 2. | 0.08 | Chloroform | 0.56 | 1:7 |
| 3. | 0.08 | Methanol | 0.56 | 1:7 |


**Figure 1: Extract weight and extract yield produced by various solvents**

**Table 2: Results of phytochemical analysis**

| Sr. no. | Phytochemicals | Tests | AVPE | AVCE | AVME |
|---|---|---|---|---|---|
| 1 | Alkaloids | Mayer's Test | - | - | - |
| | | Wagner's Test | + | - | - |
| 2 | Carbohydrates | Benedict's Test | + | - | - |
| | | Molish's Test | - | + | + |
| 3 | Fixed oils and fats | Spot test | + | + | + |
| | | Saponification test | - | + | - |
| 4 | Glycosides | Borntrager's test | - | - | - |
| | | Legal's test | - | - | - |
| 5 | Phenolic compounds and tannins | Ferric chloride test | - | + | + |
| | | Lead acetate test | + | - | - |
| 6 | Steroids | Libermann-Buchard's test | + | - | - |
| | | Salkowski test | + | - | - |
| 7 | Protein | Millon's test | - | - | - |
| | | Biuret test | - | - | - |
| 8 | Flavonoids | Shinoda test | - | + | + |
| | | Sulfuric acid test | - | - | - |

*Adhatoda vasica Petroleum Ether Extract (AVPE); Adhatoda vasica Chloroform Extract (AVCE); Adhatoda vasica Methanol Extract (AVME); Positive (+) – presence; Negative (-) – absence*

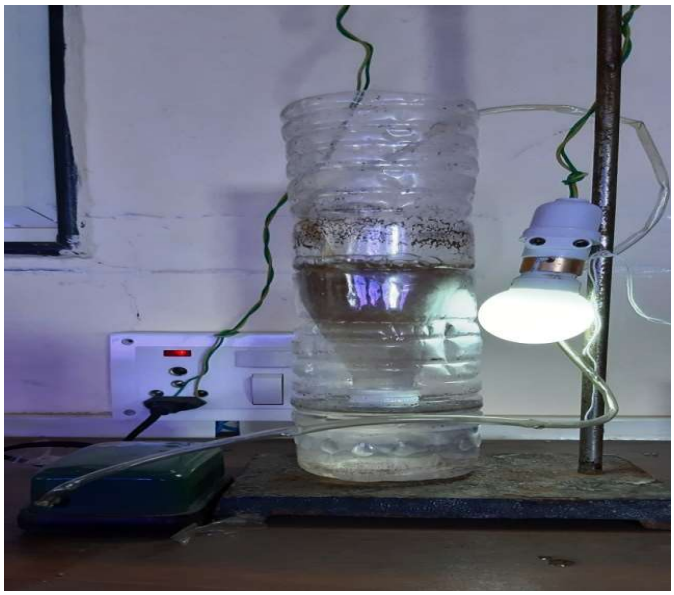
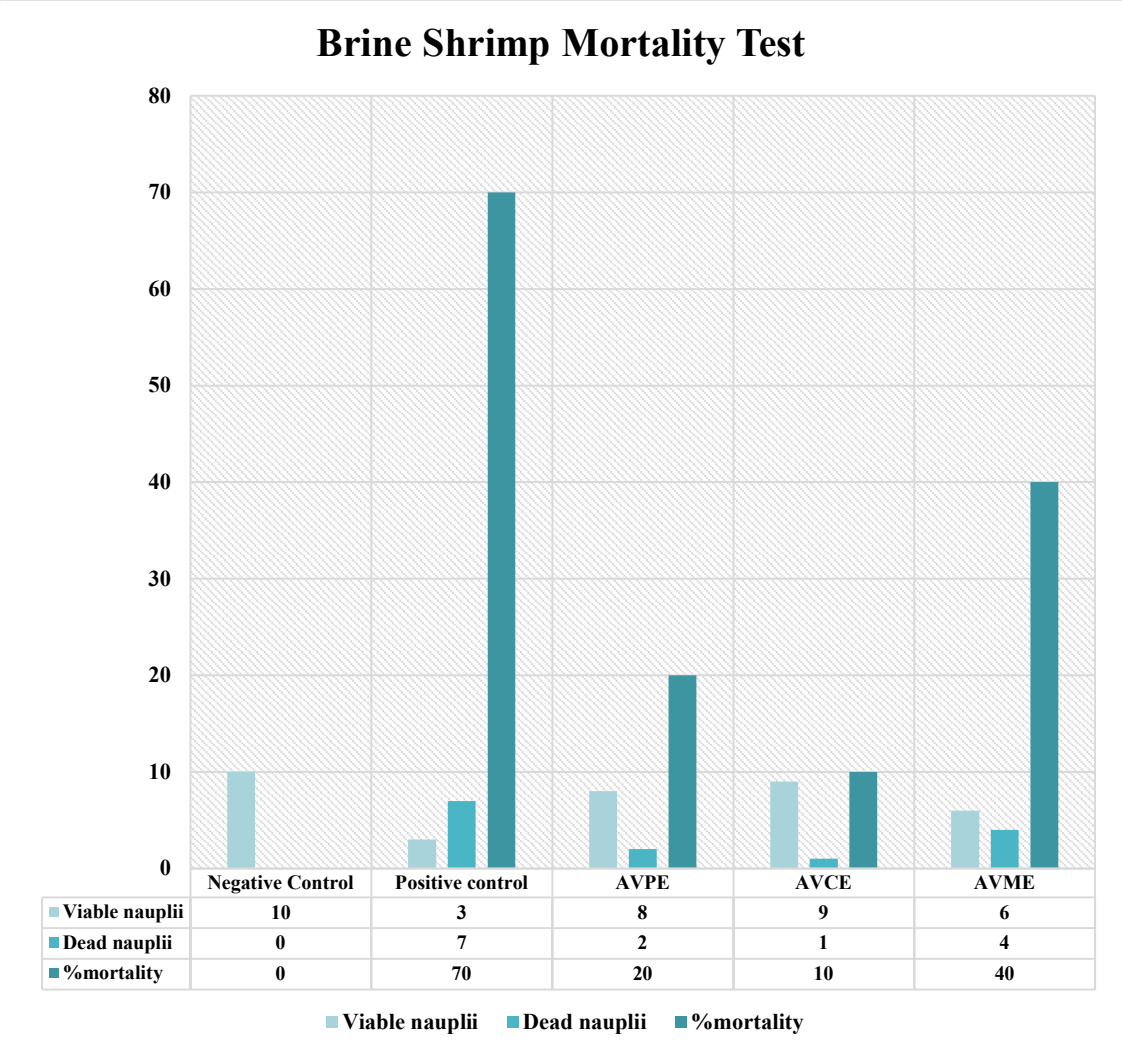


**Figure 2: Hatching of brine shrimp eggs**




| | Negative Control | Positive control | AVPE | AVCE | AVME |
|---|---|---|---|---|---|
| Viable nauplii | 10 | 3 | 8 | 9 | 6 |
| Dead nauplii | 0 | 7 | 2 | 1 | 4 |
| %mortality | 0 | 70 | 20 | 10 | 40 |

**Figure 3: Brine shrimp Mortality Test**

***Adhatoda vasica Petroleum Ether Extract (AVPE); Adhatoda vasica Chloroform Extract (AVCE); Adhatoda vasica Methanol Extract (AVME); Positive (+) – presence; Negative (-) – absence***

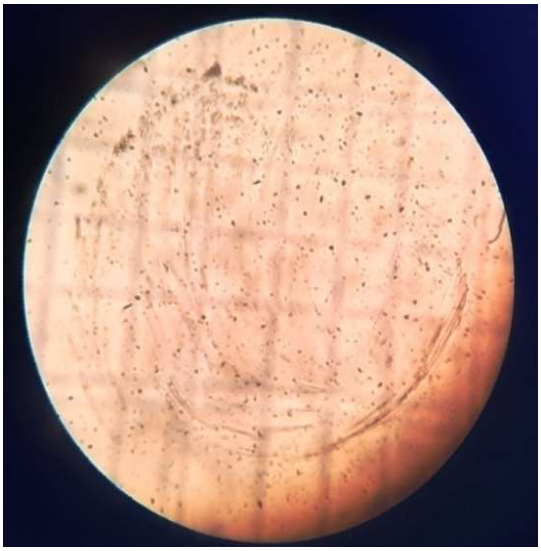


**Figure 4: *Saccharomyces cerevisiae* culture viewed under microscope**

**Table 3: Yeast cell death count**

| Sr. No. | Samples prepared | Viable cells | Dead cells | Total no. of cells |
|---|---|---|---|---|
| 1 | Negative Control | 26 | 42 | 68 |
| 2 | Positive Control | 18 | 44 | 62 |
| 3 | AVPE LD | 28 | 46 | 74 |
| 4 | AVPE HD | 23 | 43 | 66 |
| 5 | AVCE LD | 22 | 51 | 73 |
| 6 | AVCE HD | 17 | 41 | 58 |
| 7 | AVME LD | 25 | 47 | 72 |
| 8 | AVME HD | 19 | 41 | 60 |

***Adhatoda vasica*** **Petroleum Ether Extract low dose (AVPE LD);** ***Adhatoda vasica*** **Petroleum Ether Extract high dose (AVPE HD);** ***Adhatoda vasica*** **Chloroform Extract low dose (AVCE LD);** ***Adhatoda vasica*** **Chloroform Extract high dose (AVCE HD);** ***Adhatoda vasica*** **Methanol Extract low dose (AVME LD);** ***Adhatoda vasica*** **Methanol Extract high dose (AVME HD)**

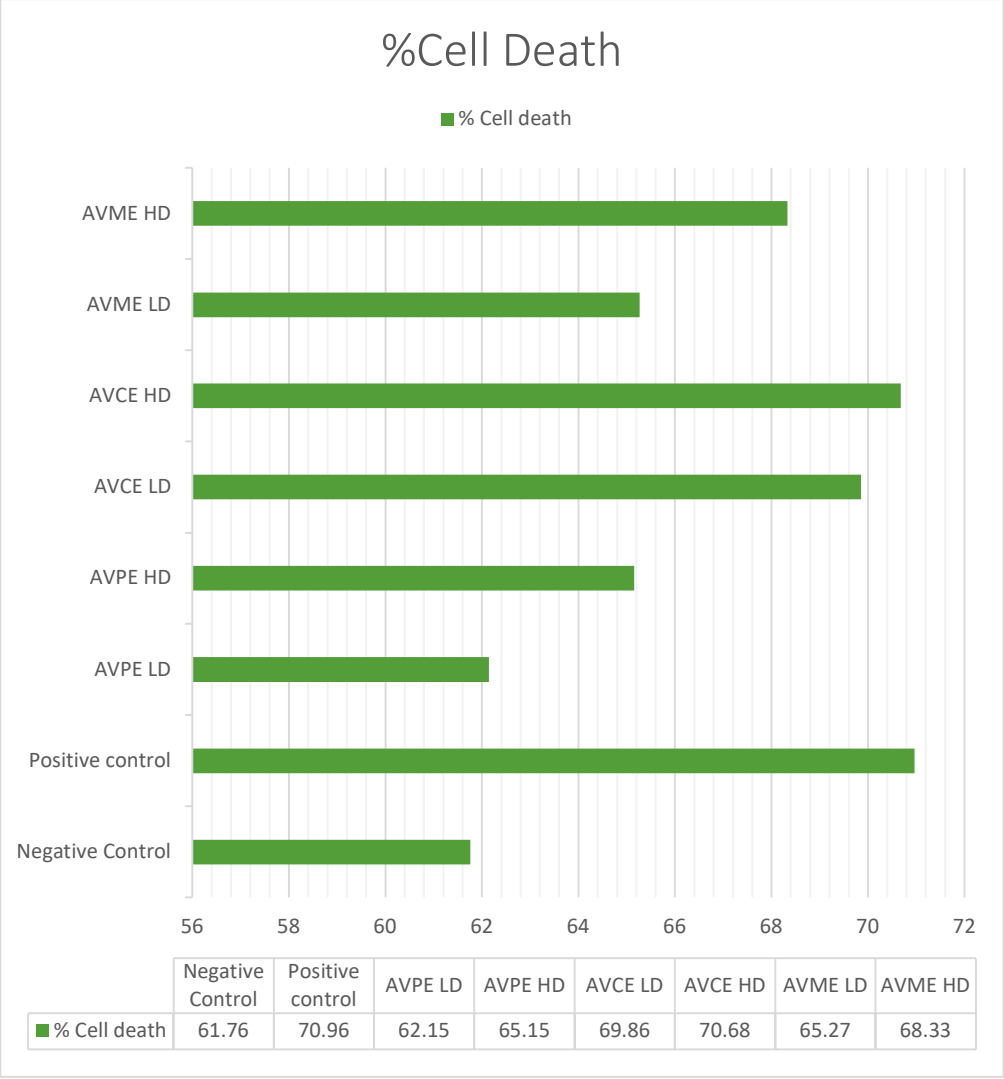



**Figure 5: % Cell Death from yeast cytotoxicity test**

***Adhatoda vasica*** **Petroleum Ether Extract low dose (AVPE LD);** ***Adhatoda vasica*** **Petroleum Ether Extract high dose (AVPE HD);** ***Adhatoda vasica*** **Chloroform Extract low dose (AVCE LD);** ***Adhatoda vasica*** **Chloroform Extract high dose (AVCE HD);** ***Adhatoda vasica*** **Methanol Extract low dose (AVME LD);** ***Adhatoda vasica*** **Methanol Extract high dose (AVME HD)**

The chloroform extract with high dose of chloroform was the most toxic towards the yeast cells (revealed by the highest calculated %cell death), among all the groups of samples treated the toxicity against *Saccharomyces cerevsisiae* was found to increase in the following order: Negative control > AVPE (LD > HD) > AVME (LD > HD) > AVCE (LD > HD) > Positive Control. Each of the three solvent extracts of *Adhatoda vasica* that were prepared, showed a toxicity level greater than 50% against the chosen test organisms. This implies that the methanol extract and chloroform extract of the herb can be further studied as a potential component to design medicinal drugs for treating parasitic and fungal infection or diseases respectively. Medicinal herbs with therapeutic effects are an important resource for drug development. Ancient Indian archives and records show the use of multiple such herbs for treating wounds, infections, ailments, and diseases. *Adhatoda vasica nees* is one of these herbs mentioned and has been used as a treatment for several medical conditions. The leaves, roots, and flowers have been used for treating gonorrhoea, respiratory distress, piles, tuberculosis, jaundice, and several more ailments. The plant expresses bronchodilation, anti-asthmatic, injury healing, anti-microbial (in particular anti-bacterial and anti-tubercular, with low levels of anti-helminthic activity), cholerectic, anti-hypersensitivity, anti-blister, ectonic, and abortion inducing activity **[24]**.

**CONCLUSION:**

The study was conducted to analyse the phytochemical components and cytotoxicity effect of the herb on brine shrimp and yeast. In the cytotoxic study using brine shrimp, the methanol extract showed high toxicity, petroleum ether extract showed moderate toxicity and chloroform extract showed least toxicity. While, in the cytotoxic study using yeast, the chloroform extract showed the highest toxicity, followed by the methanol extract and the petroleum ether extract sequentially. The present study supports that: The organic solvent extract of leaves of *Adhatoda vasica* contains medicinally important bioactive compounds that is confirmed by the phytochemical test. All the solvent extracts showed great variations for phytochemical analysis. Brine shrimp lethality bioassay and yeast cytotoxic assay are simple, reliable, convenient and inexpensive methods for monitoring and carrying out assessments of the biological activities and toxicity of various medicinal plant extracts and lends support for their use in traditional medicine.

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