CHAPTER 3: ANTIVIRAL ACTIVITY OF
PHYLLANTHUS AMARUS, BERBERINE, CURCUMIN
AND PICROLIV
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3.4. DISCUSSION
3.1. INTRODUCTION

Viruses continue to pose threat to humans and other domestic animals, the prevention and eradication of the viruses become a major challenge. Effective vaccines were developed for a very small group of viruses. Most of the antiviral drugs which are currently being used are of limited therapeutic usefulness; possess several other problems in the clinical level such as tolerability, toxicity and virus-drug resistance. Another major concern is the cost of these medicines. So there is an urgent need for more antiviral drugs as it is an issue of global concern (Clercq, 2004; Asres et al, 2005).

The plant kingdom is rich in bioactive components as well as their natural derivatives which are reported to be potent inhibitors of viruses (Subramoniam et al, 2004). Several compounds of varied chemical structures are also isolated from medicinal plants with antiviral activity (Perez, 2003). Several flavonoids like baicalin I and taxifolin, coumarins like calanolide A, terpenes such as limonoids, betulinic acid and alkaloids such as harman, manadomanzamine etc were demonstrated have potent anti-HIV activity (Asres et al, 2005).

In the present chapter we have evaluated the antiviral potential of *P. amarus*, Curcumin, Berberine and Picroliv against three viruses namely Newcastle disease virus (NDV), egg drop syndrome 1976 (EDS76) virus and Poliovirus I.

3.2. MATERIALS AND METHODS

3.2.1. Embryonated eggs:

Chick embryonated eggs and Duck embryonated eggs were obtained from the hatchery of Dept. Poultry, College of Veterinary and Animal Sciences, Mannuthy.

3.2.2. Viruses

NDV (Ranikhet Disease Strain, R2B) was initially derived from a live vaccine of Indovax Pvt Ltd, Haryana. The vaccine was passaged for 6 times in chick embryos to get a good titre stock virus. EDS 76 virus was a kind gift from Dr. G. Krishnan Nair, Dept. Microbiology, College of Veterinary and Animal Sciences, Mannuthy and Poliovirus I was kindly provided by
3.2.3. Evaluation of toxicity of Phyllanthus amarus extract, Berberine, Curcumin and Picroliv in chick embryonated eggs:

Six day old chick embryonated eggs were wiped with alcohol and kept at 39\(^\circ\)C with 60% humidity before the experiment. On day seven, eggs were candled and live eggs were taken. Following concentrations of the drugs were used for toxicity evaluation.

*Phyllanthus amarus* extract: 100, 250, 500 & 750 µg/egg (in sterile distilled water)

Berberine: 100 & 200µg/egg (in sterile distilled water)

Curcumin: 100 & 200µg/egg (Stock solution was prepared in methanol and dilutions were made in sterile distilled water)

Picroliv : 100 & 200µg/egg (in sterile distilled water).

Ten eggs were used for each concentration and one set of ten eggs were kept as normal (without any treatment). The drugs were inoculated (maximum volume used is 0.2mL) through allantoic route. After inoculation eggs were sealed and kept back in the incubator at 37\(^\circ\)C. The eggs were candled every day to check the growth of the embryo and mortality. On 13\(^{th}\) day, eggs were chilled at 4\(^\circ\)C and harvesting was done on the next day.

For harvesting eggs were open on the top and using a sterile pipette allantoic fluid was collected into sterile container. Following observations were made.

a) Growth and morphology of the embryo.

b) Morphology of Chorioallantoic Membrane (CAM)

c) Haemagglutination assay of allantoic fluid against fresh chicken blood: This assay is one of the most common methods for quantifying virus particles in a suspension. Haemagglutination is the aggregation of RBCs in the presence of heamagglutinating virus particles. For the assay fresh chicken blood was collected in Alsevier’s solution from a local slaughter house. 5% solution of RBCs was prepared and plated on a round bottom 96 well titre plate (Tarsons). 0.1mL of the
allantoic fluid was added to the first well and serial dilutions were made. The plates were then examined for haemagglutination (Burleson et al, 1992).

3.2.4. Determination of antiviral activity of Phyllanthus amarus extract, Berberine, Curcumin and Picroliv against Newcastle Disease (NDV) virus in chick embryonated eggs:

Six day old chick embryonated eggs were wiped with alcohol and kept at 39\(^0\)C with 60% humidity before the experiment. On day seven, eggs were candled and live eggs were taken. The eggs were divided into following groups (10 eggs in each group).

Group I    -  Normal (without any treatment)
Group II   -  Control (0.1mL NDV alone)
Group III   -  P.amarus 200µg/ egg + 0.1mL NDV
Group IV    -  P.amarus 100µg/ egg + 0.1mL NDV
Group V     -  P.amarus 50µg/ egg + 0.1mL NDV
Group VI    -  Picroliv 200µg/ egg + 0.1mL NDV
Group VII   -  Picroliv 100µg/ egg + 0.1mL NDV
Group VIII  -  Picroliv 50µg/ egg + 0.1mL NDV
Group IX    -  Berberine 200µg/ egg + 0.1mL NDV
Group X     -  Berberine 100µg/ egg + 0.1mL NDV
Group XI    -  Berberine 50µg/ egg + 0.1mL NDV
Group XII   -  Curcumin 200µg/ egg + 0.1mL NDV
Group XIII  -  Curcumin 100µg/ egg + 0.1mL NDV
Group XIV   -  Curcumin 50µg/ egg + 0.1mL NDV

After inoculation eggs were sealed and kept back in the incubator at 37\(^0\)C. The eggs were candled every day for mortality. On 13\(^{th}\) day, eggs were chilled at 4\(^0\)C and harvesting was done on the next day.

For harvesting eggs were open on the top and using a sterile pipette allantoic fluid was collected into sterile container. Following observations were made.

a) Growth and morphology of the embryo: After collecting allantoic fluid the embryo was taken into a sterile petri dish and abnormalities were
scored. The inhibition of growth by the embryo and other morphological abnormalities were scored and compared the same between normal untreated, virus alone treated and virus + drug treated embryos.

b) Morphology of CAM: The morphology of the CAM was evaluated

c) Haemagglutination assay of allantoic fluid against chicken blood:

3.2.5. Determination of antiviral activity of Phyllanthus amarus extract, Berberine, Curcumin and Picroliv against EDS76 virus in Duck embryonated eggs:

Six day old duck embryonated eggs were wiped with alcohol and kept at 39°C with 60% humidity before the experiment. On day seven, eggs were candled and live eggs were taken. The eggs were divided into following groups (10 eggs in each group).

- **Group I** - Normal (without any treatment)
- **Group II** - Control (0.1mL EDS76 virus alone)
- **Group III** - *P. amarus* 200µg/egg + 0.1mL EDS76 virus
- **Group IV** - *P. amarus* 100µg/egg + 0.1mL EDS76 virus
- **Group V** - *P. amarus* 50µg/egg + 0.1mL EDS76 virus
- **Group VI** - Picroliv 200µg/egg + 0.1mL EDS76 virus
- **Group VII** - Picroliv 100µg/egg + 0.1mL EDS76 virus
- **Group VIII** - Picroliv 50µg/egg + 0.1mL EDS76 virus
- **Group IX** - Berberine 200µg/egg + 0.1mL EDS76 virus
- **Group X** - Berberine 100µg/egg + 0.1mL EDS76 virus
- **Group XI** - Berberine 50µg/egg + 0.1mL EDS76 virus
- **Group XII** - Curcumin 200µg/egg + 0.1mL EDS76 virus
- **Group XIII** - Curcumin 100µg/egg + 0.1mL EDS76 virus
- **Group XIV** - Curcumin 50µg/egg + 0.1mL EDS76 virus

After inoculation eggs were sealed and kept back in the incubator at 37°C. The eggs were candled every day for mortality. On 13th day, eggs were chilled at 4°C and harvesting was done on the next day. The harvesting was done as described in the section 3.2.4. Fresh duck blood was used for the determination of haemagglutination titre.
3.2.6. Evaluation of cytotoxicity of Phyllanthus amarus extract, Curcumin, Berberine, and Picroliv against Vero cells using MTT assay

The antiviral activity of Phyllanthus amarus extract, Berberine, Curcumin and Picroliv against Polio virus is being done in Vero cells. In order to check the toxicity of above drugs against Vero cells we performed this experiment.

Vero cells (5000 cells/well) were plated on a 96-well titre plate and kept at incubator at 37°C with 5% CO₂. After 24 hr, wells were washed with PBS and various concentrations of the drugs were added. The concentrations used in the study were 5-100µg/mL for P.amarus, Berberine and Picroliv and 1-6µg/mL for Curcumin. Plates were then further incubated for 48hr at 37°C with 5% CO₂. The 50% cytotoxic concentrations were determined using MTT assay.

3.2.7. Determination of antiviral activity of Phyllanthus amarus extract, Berberine, Curcumin and Picroliv against Poliovirus I in Vero cell line:

The confluent monolayer of Vero cells was washed twice with PBS and 0.2mL of virus suspension was added to the monolayer incubated for 90 min at 37°C with occasional shaking. After the incubation monolayer was again washed with PBS to remove the unadsorbed virus and 5mL of the DMEM with 10% FCS was added and incubation continued for 16-18 hr at 37°C. After that the flask was subjected to 3 quick freeze and thaw cycles to liberate the viral particles from the cells. The supernatant was collected and centrifuged at 3000 rpm for 5 min to settle down the cell debris. The clear supernatant was aliquoted and kept at -70°C. The TCID₅₀ of the stock virus was found to be 10⁻⁶/mL.

For determining the antiviral activity Vero cells (5000 cells/well) were plated on a 96-well titre plate and kept at incubator at 37°C with 5% CO₂. After 24 hr, wells were washed with PBS and Poliovirus I (TCID₅₀ - 10⁻⁶/mL) was added to each well and allowed to adsorb for 90 min at 37°C with occasional shaking. The residual virus was washed off with PBS. P.amarus
(1-50 \mu g/mL), Berberine (2.5-25\mu g/mL), Picroliv (2.5-25\mu g/mL) and Curcumin (0.1- 2 \mu g/mL) were added to the well and plate was further incubated at 37^{0}\text{C} with 5\% CO_2. After 48^{th} hr viral inhibition rate was calculated using MTT assay as described by Guo et al (2006)

\text{Viral inhibition rate} = \frac{(\text{OD}_{tv}-\text{OD}_{cv})}{(\text{OD}_{cd}-\text{OD}_{cv})} \times 100\%

Where \text{OD}_{tv} - absorbance of the test compounds with virus infected cells
\text{OD}_{cv} - absorbance of the virus control
\text{OD}_{cd} - absorbance of the cell control

The selectivity index (SI) was calculated as the ratio of CC_{50} (50\% cell cytotoxic concentration) to EC_{50} (50\% effectiveness concentration)

3.3. RESULTS
3.3.1. Evaluation of toxicity of \textit{P.amarus} extract, Berberine, Curcumin and Picroliv in chick embryonated eggs:

None of the compounds produced any kind of toxicity to chick embryos at the concentrations given above. No mortality was observed in any of the concentrations. Growth was similar to that of untreated embryos. CAM did not show any kind of toxicity. Haemagglutination titre did not show any agglutination. The study clearly confirmed the non toxicity of \textit{P.amarus} extract, Berberine, Curcumin and Picroliv to chick embryos at the concentrations studied.

3.3.2. Determination of antiviral activity of \textit{P.amarus} extract, Berberine, Curcumin and Picroliv against Newcastle Disease (NDV) virus in chick embryonated eggs:

\textit{P.amarus} extract, Berberine, Curcumin and Picroliv showed significant antiviral activity against Newcastle Disease virus. In NDV alone treated embryos virus produced significant inhibition of growth. Embryos were poorly developed and were associated with hemorrhage and developmental abnormalities. Treatment of embryos with above drugs reversed the NDV induced cytopathic changes. Higher concentration treated (200\mu g/ egg) embryos almost looked alike the normal.

The haemagglutination assay was done against chicken RBCs. The virus alone treated group had a titre of 1024. The administration of
*P. amarus* extract at a concentration of 200µg/egg reduced the titre to 4. Almost similar results were obtained with Picroliv, Berberine and Curcumin. Out of the four drugs Picroliv showed maximum activity at the concentrations studied here. The results of haemagglutination titre were given in Table 3.1.

### 3.3.3. Determination of antiviral activity of *P. amarus* extract, Berberine, Curcumin and Picroliv against EDS76 virus in Duck embryonated eggs:

All these compounds showed significant antiviral activity against EDS76 virus also. The treatment with these compounds reversed the virus induced cytopathic changes. The heamagglutination titre was determined using fresh duck blood. The virus alone groups had a titre value of 4096. The titre values were decreased in drugs treated groups. *P. amarus* at a concentration of 200µg/egg showed maximum decrease in the value which was 4. The heamagglutination titre values were given in Table 3.2.

### 3.3.4. Evaluation of cytotoxicity of *P. amarus* extract, Berberine, Curcumin, and Picroliv against Vero cells using MTT assay:

The concentrations used here are found to be moderately cytotoxic to Vero cells. *P. amarus* at a concentration of 100µg/mL produced a cytotoxicity of 33.72%, Picroliv at a concentration of 100µg/mL produced a cytotoxicity of 30.17% where as Curcumin at a concentration of 6µg/mL produced a cytotoxicity of 30.67% as given in Figure 3.1 and 3.2.

### 3.3.5. Determination of antiviral activity of Phyllanthus amarus extract, Berberine, Curcumin and Picroliv against Poliovirus I in Vero cell line:

All the drugs showed significant antiviral activity against Poliovirus I. For *P. amarus* at a concentration of 50µg/mL the viral inhibition rate was 80.95% (Figure 3.3). Curcumin was found to be most effective and at very low concentration of 2µg/mL it produced a viral inhibition rate of 73.46% (Figure 3.4). Both Picroliv and Berberine was also found to inhibit the growth of the virus and results were given in Figure 3.5.
EC\textsubscript{50} and Selectivity index of *P. amarus*, Curcumin, Picroliv and Berberine were given Table 3.3. Out the four compounds, at the concentration studied *P. amarus* showed maximum selectivity index.

**3.4. DISCUSSION**

Newcastle disease was first reported from Tamil Nadu by Kylasamaier (1931). The disease is a highly contagious viral disease of poultry affecting chickens, pigeons and many wild birds of all ages worldwide. NDV or avian paramyxovirus 1 is the causative organism (a type of RNA virus) and it belonging to the family Paramyxoviridae. The mortality of the affected flock is nearly 90% resulting in heavy economic losses (Nanthakumar et al, 2000). Infection with NDV is characterized by respiratory distress, depression, diarrhea, impairment of central nervous system, morbidity and decreased egg production. Based on their pathogenecity NDVs have been classified as lentogenic, mesogenic and velogenic phenotypes (Collins et al, 1993).

Egg drop syndrome 1976 is an economically important disease characterized by a severe and sudden drop in egg production with a high percentage of shell defects and the causative agent, EDS virus is the sole member of group III avian adenoviruses (Senthilkumar et al, 2004). Poliovirus is an enterovirus cause a paralytic disease (poliomyelitis). There are global efforts to eradicate poliomyelitis using several approaches.

Embryonated egg techniques are generally used for the study of avian viruses. In the present investigation all the compounds did not show any toxicity to embryonated eggs even at a concentration of 200µg/egg. They also found to inhibit the growth of both NDV and EDS. The exact mechanism of their action is not known. Probably these compounds are interfering with the replication of the viruses in the allantoic cavity.

These compounds were also found to inhibit the propagation of Poliovirus in culture. The EC\textsubscript{50} was lowest for Curcumin and the selectivity index was highest for *P. amarus*.

*P. amarus* is known as an antiviral agent especially against HBV and HCV (Blumberg et al, 1989; Bhattacharyya et al, 2003). Some of the
individual components isolated from *P. amarus* like phyllanthin and repandusinic acid A monosodium salt (RA) (Ogata et al, 1992) were known for its antiviral activity. A recent report of based on the expression microarray analysis on the antiviral activity of another *Phyllanthus* subspecies, *Phyllanthus nanus* showed that the anti HBV action of *P. nanus* is mediated mainly via the over expression of annexin 7 protein and it could cause a decrease in the secretion of HBsAg (Lam et al, 2006).

Further investigations are required to evaluate the mechanism through which *P. amarus*, Curcumin, Picroliv and Berberine mediate its antiviral activity. Moreover use of these inexpensive phytochemicals in the control of viral diseases in poultry needs further evaluation.
Figure 3.1: Cytotoxicity of *P. amarus*, Picroliv and Berberine against Vero cell line

![Graph showing cytotoxicity of P. amarus, Picroliv, and Berberine. The x-axis represents concentration (µg/mL) ranging from 5 to 100. The y-axis represents % of cytotoxicity ranging from 0 to 40. The graph includes bars for each concentration level, with different colors for each compound: P. amarus (brown), Picroliv (purple), and Berberine (yellow). The error bars indicate variability.](image-url)
Figure 3.2: Cytotoxicity of Curcumin against Vero cell line
Figure 3.3: Antiviral activity of *P. amarus* against Poliovirus I
Figure 3.4: Antiviral activity of Curcumin against Poliovirus I
Figure 3.5: Antiviral activity of Berberine and Picroliv against Poliovirus I

![Bar chart showing antiviral activity of Berberine and Picroliv against Poliovirus I](chart.png)

- **Y-axis**: Viral inhibition rate
- **X-axis**: Concentration (µg/mL)
- **Legend**:
  - Orange bars: Berberine
  - Purple bars: Picroliv
Table 3.1: Effect of *P. amarus*, Picroliv, Berberine and Curcumin on HA titre in NDV inoculated embryonated eggs

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TITRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I - Normal (without any treatment)</td>
<td>0</td>
</tr>
<tr>
<td>Group II - Control (0.1mL NDV alone)</td>
<td>1024</td>
</tr>
<tr>
<td>Group III - <em>P. amarus</em> 200µg/ egg + 0.1mL NDV</td>
<td>4</td>
</tr>
<tr>
<td>Group IV - <em>P. amarus</em> 100µg/ egg + 0.1mL NDV</td>
<td>8</td>
</tr>
<tr>
<td>Group V - <em>P. amarus</em> 50µg/ egg + 0.1mL NDV</td>
<td>32</td>
</tr>
<tr>
<td>Group VI - Picroliv 200µg/ egg + 0.1mL NDV</td>
<td>2</td>
</tr>
<tr>
<td>Group VII - Picroliv 100µg/ egg + 0.1mL NDV</td>
<td>8</td>
</tr>
<tr>
<td>Group VIII - Picroliv 50µg/ egg + 0.1mL NDV</td>
<td>16</td>
</tr>
<tr>
<td>Group IX - Berberine 200µg/ egg + 0.1mL NDV</td>
<td>2</td>
</tr>
<tr>
<td>Group X - Berberine 100µg/ egg + 0.1mL NDV</td>
<td>8</td>
</tr>
<tr>
<td>Group XI - Berberine 50µg/ egg + 0.1mL NDV</td>
<td>32</td>
</tr>
<tr>
<td>Group XII - Curcumin 200µg/ egg + 0.1mL NDV</td>
<td>4</td>
</tr>
<tr>
<td>Group XIII - Curcumin 100µg/ egg + 0.1mL NDV</td>
<td>16</td>
</tr>
<tr>
<td>Group XIV - Curcumin 50µg/ egg + 0.1mL NDV</td>
<td>32</td>
</tr>
</tbody>
</table>
**Table 3.2: Effect of *P. amarus*, Picroliv, Berberine and Curcumin on HA titre in EDS76 virus inoculated embryonated eggs**

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TITRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I: Normal (without any treatment)</td>
<td>0</td>
</tr>
<tr>
<td>Group II: Control (0.1mL EDS76 virus alone)</td>
<td>4096</td>
</tr>
<tr>
<td>Group III: <em>P. amarus</em> 200µg/egg + 0.1mL EDS76</td>
<td>4</td>
</tr>
<tr>
<td>Group IV: <em>P. amarus</em> 100µg/egg + 0.1mL EDS76</td>
<td>8</td>
</tr>
<tr>
<td>Group V: <em>P. amarus</em> 50µg/egg + 0.1mL EDS76</td>
<td>16</td>
</tr>
<tr>
<td>Group VI: Picroliv 200µg/egg + 0.1mL EDS76</td>
<td>16</td>
</tr>
<tr>
<td>Group VII: Picroliv 100µg/egg + 0.1mL EDS76</td>
<td>16</td>
</tr>
<tr>
<td>Group VIII: Picroliv 50µg/egg + 0.1mL EDS76</td>
<td>32</td>
</tr>
<tr>
<td>Group IX: Berberine 200µg/egg + 0.1mL EDS76</td>
<td>8</td>
</tr>
<tr>
<td>Group X: Berberine 100µg/egg + 0.1mL EDS76</td>
<td>16</td>
</tr>
<tr>
<td>Group XI: Berberine 50µg/egg + 0.1mL EDS76</td>
<td>32</td>
</tr>
<tr>
<td>Group XII: Curcumin 200µg/egg + 0.1mL EDS76</td>
<td>16</td>
</tr>
<tr>
<td>Group XIII: Curcumin 100µg/egg + 0.1mL EDS76</td>
<td>16</td>
</tr>
<tr>
<td>Group XIV: Curcumin 50µg/egg + 0.1mL EDS76</td>
<td>32</td>
</tr>
</tbody>
</table>
Table 3.3: EC$_{50}$ and Selectivity index of *P. amarus*, Curcumin, Picroliv and Berberine against Poliovirus I

<table>
<thead>
<tr>
<th>Drug</th>
<th>CC$_{50}$ (µg/mL)</th>
<th>EC$_{50}$ (µg/mL)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. amarus</em></td>
<td>&gt;100</td>
<td>5.40</td>
<td>18.51</td>
</tr>
<tr>
<td>Curcumin</td>
<td>&gt;6</td>
<td>0.40</td>
<td>15.00</td>
</tr>
<tr>
<td>Picroliv</td>
<td>&gt;100</td>
<td>10.80</td>
<td>9.25</td>
</tr>
<tr>
<td>Berberine</td>
<td>&gt;100</td>
<td>7.42</td>
<td>13.47</td>
</tr>
</tbody>
</table>