Anti-Rotaviral Effects of Bauhinia Variegata methanolic extract in Mice with Rotavirus Diarrhea

1Mohamed Shaheen, 2Mamdouh El-Gamal, 2Adel Mousa, 3Samy Mostafa, 1Nagwa El-Esnawy

1Department of Water Pollution Research, National Research Center, Dokki, Cairo, Egypt.
2Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Cairo, Egypt.
3Department of Medicinal and Aromatic Plants Research, National Research Center, Dokki, Cairo, Egypt.

ABSTRACTS

Background. Since Rotavirus is one of the leading pathogens that cause severe gastroenteritis and represents a serious threat to human and animal health, researchers have been searching for cheap, safe, and effective anti-rotaviral drugs. There is a wide spread of interest in using natural products a sanitviral agents. Recently, Bauhinia variegata has been reported to have anti-inflammatory. Objective: The aim of this study was to evaluate the in vivo anti-rotavirus properties of methanolic extract of Bauhinia variegata leaves. The antiviral activity in vivo was based on the determination of mortality, severity diarrhea with duration of recovery, virus titers and lesion scores of intestinal tract of treated mice. Results: Our results demonstrated that the methanolic extract was safe in mice without mortality at a dose of 100 mg/kg/day. Therefore we used 100 and 50 mg/kg/day of methanolic extract to be evaluated against RV infection in mice. Our results suggested that the methanolic extract at the both dosages reduced the mortality, virus titers, severity diarrhea with duration of recovery, and intestinal lesion when compared with those in untreated control. Conclusion: The methanolic leave extract of B. variegata showed potential antiviral activity against RV infection in vivo and may be useful agent in the treatment of RV induced diarrhea.

Key words: Rotavirus, Bauhinia variegata, in vivo, cytotoxicity, antiviral drug.

Introduction

Group A rotaviruses (RVAs) are the leading cause of gas troenteritis is, malnutrition, and diarrhea in young children and drenand animals (Boshuizen, 2003). Rotavirus (RV) is a leading cause of acute gastroenteritis (AGE), affecting 95% of children below five years of age (Anca et al., 2014). Rotavirus is the leading cause of severe diarrhea among children<5 years worldwide. Currently licensed rotavirus vaccines have been efficacious and effective, with many countries reporting substantial declines in diarrhea and rotavirus-specific morbidity and mortality. However, the full public health impact of these vaccines has not been realized. Most countries, including those with the highest disease burden, have not yet introduced rotavirus vaccines into their national immunization programs (Yen et al., 2014). Deaths from RVA infection are most prevalent in developing nations, where patients may not always receive adequate medical attention quickly enough (Pesavento et al., 2006).

Currently, there are two vaccines available in the market for clinical use, RotaTeq (Merck) and Rotarix (GlaxoSmithKline) (Anderson et al., 2008). These vaccines appear to be promising in preventing RVA diarrhea. However, each is only effective against a particular strain of the virus, the high cost of production, and has a high probability of manifesting side effects in particular with vaccine-derived transmission of RVA simmmununocompromised patients. Insipite of these vaccines have the ability to prevent and control rotavirus diarrhea however it they act against certain strains of rotavirus and beside their high cost they also not completely safe in immuno-compromised persons (Baek et al., 2010).So, it is urgent to develop a new effective, cheap, and safe drug to prevent rotavirus diarrhea and therefore this was the aim from the present study.

Bauhinia variegata L., belonging to the family of Fabaceae, is a medium-sized tree, and sometimes known as Kachnar. It used in the treatment of bronchitis, leprosy, tumors, and ulcers (Kirtikar and Basu, 1993). Recently, B. variegata has been reported to have antidiabetic (Kumar et al., 2012), anti-inflammatory (Mohamed et al., 2009), antiobesity (Balamurugan and Muralidharan, 2010), antioxidant activity (Mishra et al., 2013; Rajani and Ashok 2009), antimicrobial activity (Gunalan et al., 2011; Rasheed et al., 2013), nephroprotective (Prusty et al., 2012), hepatoprotective (Marasani, 2014), anticancer activities (Mishra et al., 2013).

Constituents isolated from the leaves of B. variegata were included lupeol, alkaloids, fat glycoside, oil, phenolics, lignin, saponins, β-sitosterol, terpinoids, tannins, rutin, kaempferol-3-glucoside, quercetin, quercitrin, reducing sugars, apigenin-7-O-glucoside, apigenin, amides, carbohydrates, protein, fibers, vitamin C, phosphorus, and calcium (Anonymous, 1998; Dhale, 2011; Sharma, 1966; Sharma, 1968 and Spilkova, 1992).

Corresponding Author: Mohamed Shaheen, Department of Water Pollution Research, National Research Center, Dokki, Cairo, Egypt.
E-mail: m_nrc2007@yahoo.com
The aim of this study was to evaluate the in vivo anti-rotavirus properties of crude extracts of methanolic extract of *Bauhinia variegata* leaves. The antiviral activity in vivo was based on the determination of mortality, severity diarrhea with duration of recovery, virus titers and lesion scores of intestinal tract of treated mice.

**Material and Methods**

**Plant collection:**

*Bauhinia variegata* leaves were collected from the Botanical Garden of the National Research Centre (NRC), Cairo, Egypt during May and June 2011 and was kindly identified by, Mrs. Tersea Labib, taxonomist at Orman botanical garden, Giza and Dr. Mona Marzok, Researcher in NRC.

**Preparation of Extracts:**

*Bauhinia variegata* leaves were shade dried, ground and fine powdered then extracted by percolation with methanol at room temperature and concentrated under reduced pressure to dryness to yield methanol extract of *B. variegata* leaves (yield 16.66%).

**Cells and virus:**

The adherent monkey kidney cell line MA-104 clone 1 (ATCC CRL-2378) was used to support the growth of RV. Briefly, MA-104 cells were propagated in monolayer cultures using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100µg/ml streptomycin to mycin under 5% CO2 humidified incubator. The titers of RV SA11 was determined by standard Karber method (Karber, 1931), then stored in small aliquots at – 80 °C until used.

**In vivo experiments:**

**Cytotoxicity determinations:**

Acute toxicity of Mathanolic extract of *B. variegata* in newborn mice was determined by orally inoculating mice that are 1 weeks old and at weight ranges 10-14 g with *B. variegata* extract ranging from, 400, 300, 200, and 100 mg/kg/day/mice for seven consecutive days. The mortalities were observed daily for 3 weeks. The mortality rate of newborn mice at different Mathanolic extracts of *B. variegata* concentrations was recorded to calculate the LD50 of Mathanolic extract of *B. variegata* of newborn mice over treatment for seven consecutive days.

**Antiviral activity:**

**Experimental design:**

Forty five Newborn BALB/c male mice, (1 weeks old and weight ranges 10-14g) obtained from animal house of National Research Center in Cairo were used in this study. Thirty seven mice were orally inoculated with activated RV SA11 at 10^6 TCID50 in a volume of 100 µl via oral gavage. Thirty two of developed diarrhea mice were selected and randomly divided into four groups (8 mice each group). Three groups of inoculated mice were treated orally by extracts separately at two concentrations (100 and 50 mg/kg body) or injected intra-peritoneal with ribavirin at a dose of 10 mg/kg (positive control) daily for 7 days and observed carefully. Eight inoculated mice were treated with an oral dose of 0.9% saline solution daily and used as infected control. Uninoculated mice (n = 8) were treated with 0.9% saline solution daily and used as normal control.

**Diarrhea and severity of illness scoring:**

To determine the ability of the methanolic extract of *B. variegata* for prevention of diarrhea resulted from rotavirus infection, after 24 h of RV inoculation, each extract was given orally by a gavage daily for 7 days. The presence of diarrhea was determined daily for each group, and the Severity of diarrhea was evaluated as described by Tam and Michael, (2011).
Viral shedding of RV in feces:

After 24 h of rotavirus inoculation, the feces of each group were collected and pooled each day, then stool specimens were made up to 10% (w/v) suspensions in phosphate-buffered saline and after centrifugation at 3000×g for 20 minutes, approximately 200 μl supernatant containing the virus was transferred into fresh tube and mixed with 2 μl of trypsin followed by 45 min in a 37°C water bath. Ten-fold serially diluted with FBS free DMEM was prepared and 1 ml of each virus dilution was used to inoculate MA104 cell monolayers in duplicate and the virus titer was determined by a plaque assay as described by Arnold et al. (2009).

Effect of the methanolic extract on RV-induced histological alterations in the small intestine:

After 7 days from RV inoculation, four mice in each group were killed to determine the morphological changes in the small intestines. The intestinal tracts were removed from the abdominal cavities and fixed in 10% buffered formalin solution. Sectioned at 4 μm and stained with hematoxylin-eosin (HE), and examined microscopically. The average villi/crypt (V/C) ratio was used to estimate the scores of the small intestinal changes, which was measured as described previously by Kim et al. (2011). The remaining four mice of each group were used for evaluation of mortality in each group.

Statistical Analysis:

Quantitative data were statistically represented in terms, mean ± standard division (SD). Comparison between difference groups in the presents study was done using One-way ANOVA Test.

Statistical analysis was done with computer program SPSS (Statistical Package for Social Science) statistical program version (16.0) and a P value of ≤ 0.05 was considered significant.

Results:

Cytotoxicity determination in vivo:

Oral gavage treatment with the different concentrations of methanolic extracts of B. variegate for 7 days showed that the methanolic extract at 100 mg/kg/day did not produce any mortality and therefore it considered as safe extract at this dose in mice. The result is presented in Table 1.

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>Concentrations/kg body weight</th>
<th>Number of tested animals</th>
<th>Number of dead animals</th>
<th>Survival rate</th>
<th>Mortality rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00</td>
<td>8</td>
<td>0.00</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Methanolic extract of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bauhinia variegata</td>
<td>100 mg</td>
<td>8</td>
<td>0</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>200 mg</td>
<td>8</td>
<td>2</td>
<td>75%</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>300 mg</td>
<td>8</td>
<td>2</td>
<td>75%</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>400 mg</td>
<td>8</td>
<td>3</td>
<td>62.5%</td>
<td>37.5%</td>
</tr>
</tbody>
</table>

Antiviral activity against RV in vivo:

Diarrhea and severity of illness score of RV infected mice:

Diarrhea and severity of illness was checked daily after 1 day post-infection until the 7th day. As depicted in Table 2 and Figure 2, the treated groups characterized by low severity diarrhea than untreated infected control and they recovered from diarrhea in period shorter than those in untreated infected control.

Mortality:

After inoculation of mice with rotavirus, they became morbid and the mortality was recorded from 1 day post-infection until 14 days of experiment. The mortality was observed on day 10. The mortality was higher in untreated infected control (75%) than RV group (50%) and methanolic extract of B. variegata at a dose of 50 mg/kg body weight (25%). However, there was no any deaths in the group treated with the methanolic extract of B. variegata at a dose of 100 mg/kg body weight (Table 2; Figure 1).
3. Virus titers in animal feces:

The virus titers of the feces in methanolic extract or ribavirin treated groups were much lower than those of the untreated control group. These results indicate that the methanolic extract of *B. variegata* have potent activity against rotavirus replication in infected mice. (Table 2; Figures 4 and 5).

Viral shedding in the feces was detected by measuring the infectious viral particles shed at day post-inoculation 7. Our results demonstrated that the treated groups at a dose of 100 and 50 mg/kg body weight/day revealed a rapid decrease of viral shedding in fecal samples, compared with untreated infected control. We observed that the methanolic extract of *B. variegata* decreased the viral shedding by 74% and 69% at a dose of 100 and 50 mg/kg body weight respectively.

![Fig. 1](image1.jpg)

**Fig. 1:** The percent of mortality in normal control, A; Infected control, B; RBV, C; *B. variegata* methanolic extract 100 mg/kg, D; *B. variegata* methanolic extract 50 mg/kg, E; (n=4 for each group).

![Fig. 2](image2.png)

**Fig. 2:** Score and duration of diarrhea until 7th day of experiment in different groups of mice. n=8 for each group, *P* < 0.01 Scores of diarrhea (based on four points) with duration of diarrhea from 24 h post-infection versus the RV-infected group.
Fig. 3: Virus titers of the feces sampled from normal control, A; Infected control, B; RBV, C; B. variegata methanolic extract 100 mg/kg, D; B. variegata methanolic extract 50 mg/kg, E. Mice were sacrificed at day 7, and the feces of each group were homogenized and virus titers were determined as described in Materials and Methods. n = 4 for each group. *P < 0.01 versus the RV-infected group.

Fig. 4: Scores of histopathological changes in the small intestine collected from normal control, A; Infected control, B; RBV, C; B. variegata methanolic extract 100 mg/kg, D; B. variegata methanolic extract 50 mg/kg, E.

Table 2: Results of severity and duration of diarrhea, virus titers, lesion score of small intestine, and mortality in different groups of mice.

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>Mortality</th>
<th>Number of mice developed diarrhea</th>
<th>City of diarrhea scores ±S.D.</th>
<th>Duration of diarrhea (Days ± S.D.)</th>
<th>Virus Titers (log10 PFU/ml, means± SD)</th>
<th>Lesion score (means± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control group</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Infected group</td>
<td>75%</td>
<td>8</td>
<td>4±0.03</td>
<td>6.2±0.05</td>
<td>6.50 ±0.05</td>
<td>3.75±0.01</td>
</tr>
<tr>
<td>Ribavirin (1 mg/mL)</td>
<td>50%</td>
<td>8</td>
<td>3.87±0.02**</td>
<td>3.6±0.11**</td>
<td>3.13±0.03**</td>
<td>3.0±0.07**</td>
</tr>
<tr>
<td>B. variegata methanolic extract 100 mg/kg</td>
<td>0</td>
<td>8</td>
<td>3.62±0.04**</td>
<td>2.9±0.01**</td>
<td>1.68±0.01**</td>
<td>2.0±0.05**</td>
</tr>
<tr>
<td>B. variegata methanolic extract 50 mg/kg</td>
<td>25%</td>
<td>8</td>
<td>3.75±0.01**</td>
<td>3.0±0.04**</td>
<td>1.99±0.01**</td>
<td>2.5±0.01**</td>
</tr>
</tbody>
</table>

**P<0.01 compared with the infected control
Histology:

As shown microscopically, the small intestine tissue of the RV infected mice shows severe villi atrophy and crypt hyperplasia in ileum. However the groups treated with methanolic extract of *B. variegata* plant showed significantly improvement with long and slender villi at 100 mg/kg and 50 mg/kg/day when compared with untreated infected group (Table 2; Figure 5). The *B. variegata*methanolic extract at a dose of 100 mg/kg was observed as normal control and relieved 100% of the small intestinal lesion whereas this extract at 50 mg/kg was observed to relieve 86.6% of the small intestinal lesion. (Figure 5 D & E)

![Histology images](image)

**Fig. 5:** Pathologic appearance of small intestine tissues of BALB/c mice (H&E, magnification 200 X):
A, normal control mice; B, the viral infected mice; C, RBV mice; D and E, methanolic extract of *B. variegata* at 100 mg/kg and 50 mg/kg respectively.

Discussion:

Rotavirus consider as major pathogen for gastroenteritis in infants and young children. However the diarrheal rotavirus still uncontrolled thus development a new effective drug to control rotavirus infection is urgent (Kim *et al.*, 2012). Rotavirus is the leading cause of severe diarrhea disease in newborns and young children worldwide, estimated to be responsible for approximately 600–850,000 deaths each year. This represents approximately 5% of all deaths in children younger than five years of age worldwide (Dennehy, 2008). In this study, we evaluated the antiviral activity of a natural extract of methanolic extract of *Bauhinia variegata* leaves against RV infection in vitro and we observed that the methanolic extract was the strongest than other extracts against RV infection.

Using our RV induced diarrhea model, newborn mice recovered from RV infection within five days post initiation of treatment. In this study, newborn mice were pretreated with saponin extract before RV was inoculated for five consecutive days. We have proposed that the most likely mechanism of action of the extract is through disruption of cellular membrane proteins and/or virus receptors, preventing virus infection of these cells (Roner *et al.*, 2007; Roner *et al.*, 2010). The methanolic *B. variegata* extracts may cause a reversible modification of the cell membrane or modification of the cellular endocytosis process. Our *in vivo* results can be explained by the possibility that not all cells are being “treated/coated/modified” by the *B. variegata* extract at the low concentration (100mg/kg/day/mice), hence at higher inocula, RV can still establish an infection and induce diarrhea in over half of the individuals. However, the saponin extract is quite toxic to the newborn mice when the concentration is doubled to 50mg/kg/day/mice, therefore attempts in purification of the saponin extracts to reduce toxicity would be important. It is important to note that although the incidence of diarrhea was only reduced by 50% or so in some treatments, the severity and interval of the diarrhea in the animals was greatly reduced when compared to animals that did not receive the *B. variegata*-containing extract. No animals ever died as a result of the RV-induced diarrhea. The animals that died did so within 24-36 hours of receiving the *B. variegata*extract (more than 100mg/kg body weight/day), most likely due to toxicity.

Neonatal mice were inoculated with rotavirus and the severity of illness score was recorded daily depending on the color of stool. The data of the present study suggested that the severity diarrhea in the treated groups and RV was significantly less than that those in infected control without treatment. When we compared with untreated infected control, RV relieved 3.25% of severity of illness, while the methanolic extract of *B. variegata* improved the severity of diarrhea by 9.5% and 7.5% at a dose of 100 and 50 mg/kg body weight respectively. On 4 day no signs of diarrhea observed in the treated groups indicating that the methanolic extract of *B.
variegata inhibited the virus replication and prevented rotavirus induced diarrhea. Untreated infected mice did not show any improvement in fecal consistency score until the 7th of the experiment. On the other hand, the methanolic extract of B. variegata reduced the healing time from rotavirus-induced diarrhea in treated mice when compared to untreated infected group. Where we have observed that the mean healing time in the group treated with the methanolic extract ranged from 48 to 84 hrs and Ribavirin treated group (86.4 hrs) were significantly shorter than those in the untreated infected control (148.8hrs). 

Rotavirus mainly replicate in the small intestine affecting on the villus epithelial cells of the small intestine, causing vacuolar degeneration, villi atrophy and crypt hyperplasia, which can be observed clearly in the epithelial cells of untreated and RV infected neonatal mice but these signs was not found or reduced in infected neonatal treated mice when compared with normal control which have long and slender villi with short crypts of the small intestine. The B. variegata methanolic extract at a dose of 100 mg/kg was observed as normal control and relieved 100% of the small intestinal lesion whereas this extract at 50 mg/kg was observed to relieve 86.6% of the small intestinal lesion.

The phenolic compounds have been reported to possess antiviral activity against some RNA viruses such as rabies virus (Cha‘vez et al., 2006), SARS-coronavirus (Lin et al., 2005), human immunodeficiency virus (HIV) (Hu et al., 2013), and dengue virus (Sara et al., 2011). Also the flavonoid compounds have been reported to possess antiviral activity against some RNA viruses such as rotavirus (Bae et al., 2000), coxackie virus B3 (Zhu et al., 2009), Parainfluenza-3 virus (Orhanet al., 2010), rhinoviruses belonging to enterovirus (Semplest et al., 1999), influenza A (H3N2) and B viruses (Nagai et al., 1995), and parainfluenza type 3 (Li et al., 2002), and human immunodeficiency virus (HIV) (Critchfield et al., 1996).

Therefore, we determined the presence of these compounds in the extract. The data of the present study shows that the methanolic extract of B. variegata contain 28.67 mg/100mg of phenolic compound and 4.19 mg/100 mg of flavonoid compound. Thus the antiviral activity of this extract against rotavirus replication may be attributed to the presence of these compounds

These data suggest that treatment with the methanolic extract of the B. variegata not only reduce the healing time for diarrhea after rotavirus infection, but also mainly protect the small intestine from alteration that can be occurred due to rotavirus infection. Finally, we concluded that the present study shows that the methanolic extract of B. variegata leaves can play an important role in prevention of rotavirus diarrhea.

References


