Effect Of Some Plant Extracts On Bovine Rota Virus

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ABSTRACT

Study of the antiviral effects of plant extract is aimed to discover new strategies in the treatment of different infectious diseases particularly viral diseases. Many traditional medicinal plants have been reported to posses strong antiviral activity, and some of them have already been used for treatment of human and animals against different infections. The present study involves evaluation the antiviral activity of Guava and Sage leaves extracts against Bovine Rota Virus (BRV). Test was carried out on Madine Darby Bovine Kidney (MDBK) cell line and in mice. The present study explore the strong antiviral activity of Guava extract against where BRV guava leaves extract at concentration ranged between 0.03-0.6 µg/m with IC_{50} < 0.03 µg/ml while Sage at concentration ranged between 0.03-1.2 µg/ml has no significant anti-BRV activity.

The antiviral activity of Guava was against the BRV infective cycle while it has weak anti-BRV activity at the phases of BRV replication following penetration. In conclusion it is advisable to use Guava leaves to avoid infection with BRV especially in BRV exposed animals or to use it in treatment of animals infected with BRV at early stage of the disease.

INTRODUCTION

Bovine Rota Virus is a member of family Reoviridea. The virus particle is triple layered, approximately 70 nm in diameter, its genome consists of 11 segments of double stranded RNA that code for 6 structural and 6 non structural proteins *(1)*. The infection occurs world wide among calves under 3 weeks of age causing acute gastroenteritis with morbidity and mortality rates reach 37% and 25-46% respectively *(2,3)*.

Although Rota virus vaccine is often administrated to pregnant animals to increase level of Rota virus antibodies in colostrum, neonates frequently exhibit Rota viral diarrhea which may be attributed to failure of passive transfer from dam to calf *(4)*, therefore other therapeutics need to be considered for controlling the virus infection.

Many screening efforts have been made to find antiviral agents from natural sources. Many plants are now being collected and examined in an attempt to identify possible sources of antiviral *(5)*. In the last decades a large number of a phytochemicals have been recognized as a way to control infections caused by viruses *(5,6)*. Extensive literature survey revealed that guava has a history of traditional use for a wide range of diseases. The leaves and bark of guava tree have a long history of medicinal uses that are still employed today *(7)*. Also some types of fractionated extracts of Sage showed to be antiviral against vesicular stomatitis virus *(8)*. This study determined safety, effectiveness and antiviral activity of Guava and Sage extract against BRV.

MATERIAL AND METHODS

Cell culture and virus

Madine Darby Bovine Kidney (MDBK) cell line was used for detection of antiviral and cytotoxic effect Of Guava and Sage extract. The cell line obtained from Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo

Cells were cultivated at 37°C with 5% CO_2 in Eagle's MEM medium supplemented with 10% featal calf serum plus 100 IU/ml penicillin and 100 µg/ml streptomycin

Bovine rotavirus, kindly supplied by Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt, was used to study the antiviral effect of guava and sage extracts *in vitro* (MDBK cell culture) and *in vivo* (mice).

Virus dilutions were performed in maintenance media supplemented with 2% fetal calf serum and 5 µg/ml trypsin, and virus titre was calculated ***(11)***.

Laboratory animals

Swiss albino mice were used at 9-12 days of age with average body weight 7-13 gm. Mice were housed in micro-isolation cages and used for assessment of anti BRV activity of the extract of Guava.

Plant extracts

Aqueous extract were prepared from plant leaves of Guava and Sage as follow. Ten gm of dry leaves (Guava and Sage) were added to 100 ml boiled double distilled water in beaker, covered and left till cooling, then sieved using filter paper, then filtrated with syringes milipore filter 0.2 mm , a liquated, and stored at -20°C till used.

1.*In vitro* evaluation of Guava and Sage extracts as an inhibitory agent against BRV replication in MDBK cell line culture and their Cytotoxicity:

Cytotoxicity of Guava and Sage extracts

Two fold serial dilutions (1/10, 1/20, 1/40.....1/640) of each extract were prepared in cell culture maintenance medium then added to the confluent MDBK cells monolayer in 96-well microtiter plates and incubated at 37 °C and 5% CO_2.

After 3 days incubation, cytotoxicity was determined by examing cellular morphology in situ ***(9)*** and by cell staining with trypane blue ***(10)*** blue-stained cells was scored as non viable, unstained cells as viable.

Determination of cytotoxic concentration fifty (CC_{50}) of extract was determined as the concentration that induced any deviation of the morphology than the normal control cell in 50% of MDBK cell monolayer.

Antiviral activity

The antiviral effect of the two extracts was monitored through the reduction of 100 $TCID_{50}$ of BRV in MDBK cell monolayer treated with non cytotoxic concentrations of each extract in 96 well microtitre plates.

Visual determination was used for detection of antiviral activity as inhibition of viral CPE in cell culture. The value of CPE reduction was calculated as percent compared to the virus infectivity controls.

Controls of cell survival (cells+extract) were also included. Experiment was performed in the range of non toxic concentration of the extract.

Antiviral inhibitory concentration fifty (IC_{50}) of each extract was assayed as the concentration of extract that fully inhibited virus cytopathic effect in 50% of monolayers.

Pre-infection treatment assay

Two fold serial dilutions of each extract (Guava and Sage) were prepared in maintenance medium (MM) (1/160, 1/320,.....1/2560) and added into confluent monolayers of MDBK cells and incubated for 5 hours at 37°C in 5% Co_2. At the end of the incubation period, the two extracts were removed and the cultures were rinsed with MM then inoculated with Rota virus suspensions (100 $TCID_{50}$/ml). After 2 hours of virus adsorption, culture fluid was decanted and MEM were added. Plates were incubated at 37°C in 5% Co_2 till the presence of 100% CPE in virus infectivity control cultures ***(12)***.

Post-infection treatment assay

A Rotavirus suspension (100 TCID50/ml) was inoculated into monolayer MDBK cell cultures in 96 well plates. After 2 hr., cell cultures were rinsed with MM, and two-fold serial dilutions (1/160, 1/320...1/2560) of each extract (Guava and Sage) were added. Plates were incubated at 37°C in 5% Co2 till the presence of 100% CPE in virus infectivity control culture was confirmed and then cultures fixed and stained ***(13)***.

2. *In vivo*, evaluation of the inhibitory effect of Guava extract against BRV replication in mice

Toxicity of guava extract was tested in group of mice. Each mouse was inoculated with 0.2 ml intraperitoneally (I/P) of undiluted extract once a day for 3 days, mice were housed in micro-isolation cages with daily observation for any abnormal symptoms or deaths for 10 days.

Antiviral effect of guava extract on BRV infection was carried out in groups of mice was studied. Mice used in the experiment were assigned randomly into 3 groups, ten mice/group.

Group 1: were simultaneously inoculated intraperitoneally with 100 MED50 BRV (0.2 ml/mice) and concentrated guava extract.

Group 2: the mice were inoculated once with concentrated guava leaves extract, then after 24 hrs they were injected with 100 MED50 of BRV 0.2 ml/mice (pre-infected treatment).

Group 3: the mice were inoculated with 100 MED50 of BRV (0.2 ml/mice) after 24 hrs they inoculated with concentrated guava leaves extract once a day for 3 days (post infection treatment.

In addition three groups (each of 10 mice) were used as control as follows.

Ten mice treated and non-infected (extract control).

Ten mice non-treated and infected (virus control).

Ten mice non-treated and non-infected (-ve control).

Fecal samples were collected from different groups every 24 hrs between the first day and third after infection and prepared for inoculation of MDBK cell culture to titrate the shedding of BRV.

Also, mice were observed daily for diarrhea and/or deaths and the results were recorded throughout 7 days.

Fecal sample preparation and inoculation

Fecal samples were emulsified in approximate volume of PBS (pH 7.2) containing Penicillin 1000 IU/ml and streptomycin 1000 ug/ml, then homogenized and centrifuged at 1500 xg for 10 min. The supernatant was filtered via 0.45 um Millipore filter. Samples were stored in vials at -70°c until tested ***(14)***.

Titration of BRV in mice fecal samples on MDBK cell culture

Ten fold serial dilutions (10^1:10^5) of the pooled fecal samples inoculated into confluent monolayer of MDBK cells and incubated for two hrs at 37°c in 5% CO_2, at the end of the incubation period the culture inoculums were decanted, rinsed twice and MEM was added, plates were incubated at 37°c in 5% CO_2 till the presence of CPE in the virus infectivity control and the virus titer was calculated ***(11)***.

RESULTS

Cytotoxicity of extracts using MDBK

Examination of the cytotoxicity of Guava and Sage leaf extracts was performed in the range of concentration up to 10 µg/ml

From the results presented in Table 1, CC_{50} of Guava and sage extracts was (0.6 µg/ml and 1.2 µg/ml) respectively. Our data are the mean of three independent experiments. The toxicity of extracts was accompanied by changes in cell morphology.

Pre-infection treatment assay in MDBK

The results represented in Table 2 showed that: Sage leaves extract showed no antiviral effect in comparison with positive control (BRV+ cells) as CPE remained 100% at all tested concentration.

On the other hand Guava leaves extract showed antiviral effect as no CPE was observed at all tested concentrations ($IC_{50} < 0.03$ µg/ml).

Post-infection treatment assay in MDBK

Sage leaf extract caused no reduction of CPE at any of the tested concentration indicating no inhibitory potential on virus replication while Guava leaf extract showed slightly suppressed virus replication (Table 3) ($IC_{50} > 0.6$ µg/ml).

The effect of toxicity of guava extract in mice

The result of toxicity showed that the extract can be used safely where no abnormal signs or deaths occurred among treated groups.

Simultanious, pre-infection and post-infection treatment assay in mice

Simultaniously, pre-infection and post-infection treated groups of mice had almost normal intestine in comparison to infected non-treated mice (non to mild diarrhea). Also, the result represented in Table 4 showed that the treated mice shedded lower quantities of BRV in their feaces.

Table 1. Cytotoxicity of Guava and Sage in MDBK cell culture.

Extract dilution	Fraction (ug/ml)	Survival of cells %	
		Guava	Sage
1:640	0.15	100	100
1:320	0.3	100	100
1:160	0.6	50	100
1:80	1.2	0	50
1:40	2.5	0	0
1:20	5	0	0
1:10	10	0	0

Table 2. Antiviral effect of guava and sage on BRV infectivity in MDBK cell cultures that treated with extracts preinfection.

Concentration (ug/ml)	Reduction of CPE %	
	Guava	Sage
0.03	100	0
0.07	100	0
0.15	100	0
0.3	100	0
0.6	100	0
1.2	*ND	0

Reduction of CPE (%) is calculated relative to virus infectivity control.
*ND: not done.

Table 3. Antiviral effect of guava and sage on BRV infectivity in MDBK cell cultures that treated with extracts postinfection.

Concentration (ug/ml)	Reduction of CPE %	
	Guava	Sage
0.03	0	0
0.07	0	0
0.15	0	0
0.3	0	0
0.6	10-20	0
1.2	*ND	0

*ND: not done.

Table 4. Titration of shedding BRV in mice fecal samples.

Mice groups	*DPI	Fecal samples dilutions					titer	Reduction of virus titre
		1/10	1/100	1/1000	1/10000	1/100000		
1	1	+	+	–	–	–	$10^{2.5}$	10^{2}
	2	+	+	–	–	–	$10^{2.5}$	10^{2}
	3	+	–	–	–	–	$10^{1.5}$	10^{3}
2	1	+	+	–	–	–	$10^{2.5}$	10^{2}
	2	+	–	–	–	–	$10^{1.5}$	10^{3}
	3	–	–	–	–	–	$<10^{1.0}$	$>10^{3.5}$
3	1	+	+	+	–	–	$10^{3.5}$	10^{1}
	2	+	+	–	–	–	$10^{2.5}$	10^{2}
	3	+	+	–	–	–	$10^{2.5}$	10^{2}
4	1	+	+	+	+	–	$10^{4.5}$	0
	2	+	+	+	+	–	$10^{4.5}$	0
	3	+	+	+	+	–	$10^{4.5}$	0

*DPI: day post inoculation.
1: Simultaneously inoculated with leaves extract and BRV at the same time
2: Mice inoculated with leaves extract and after 24 hr inoculated with BRV.
3: Mice inoculated with BRV and after 24 hr inoculated with leaves extract.
4: Mice inoculated with BRV only (positive control).
N.B: Control group (non treated and non infected) still normal till the end of experiment.

DISCUSSION

In this study we evaluated the cytotoxicity and antiviral activity of Guava and Sage leaf extracts in the (MDBK-BRV) model system. The results obtained in investigation of cytotoxicity showed that Guava and Sage leaf extracts were reported to have CC50 = 0.6 µg/ml and 1.2 µg/ml respectively. Similar results were reported I previous study ***(15,16)***.

The result of the investigating the antiviral activity of Sage extract showed that Sage did not reduce the CPE of BRV (Table 2) in the range of non toxic concentrations.

On the other hand, Guava leaves extract completely reduce the CPE of BRV when used preinfection in treatment of MDBK cell culture and The IC_{50} of Guava extract was < 0.03 µg/ml (Table 2), while it had a weak anti BRV effect in MDBK cell culture when used only postinfection. Previous study showed that *Psidium guajava* Linn, Leaf extract has an anticough and antimicrobial activities ***(17)***. We concluded that Guava leaves extract has anti-BRV activity while Sage has not. As the antiviral activity excists if 100% reduction of CPE is observed at subsequent two fold dilution in the range of non toxic concentration of the extract ***(18,19)***.This is may be due to that Guava leaves extract has direct intracellular selective action on some steps in BRV biosynthesis ***(20)*** which may be lead to prevention of viral attachment or penetration.

In vivo we study the availability of using plant extract as preventive or therapeutic mean for BRV infection in mice. Toxicity studies in mice showed that guava leaf extract is safe without any side effects ***(21)***.

Simultaneously, pre-infection and post-infection treated groups of mice had almost normal intestine in comparison to infected non-treated mice (no to mild diarrhea). Also, the result represented in Table 4 showed that the treated mice shedding lower quantities of BRV in their feaces if compared with positive control.

These results in mice confirm the previous result in MDBK cells consequently guava leaves extract has anti BRV activity.

In conclusion Guava leaves extract has very strong antiviral activity against BRV while Sage leaves extract has no anti-BRV activity. The antiviral activity of Guava leaves extract was against the whole Rota infective cycle so, it is advisable to use Guava leaves extract as food additives to avoid infection with BRV specially in a BRV exposed animals or to use it in treatment of animals infected with BRV at early stage of the disease.

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الملخص العربى

تأثير بعض المستخلصات النباتية على فيروس روتا الأبقار

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المعمل المركزى للرقابة على المستحضرات الحيوية البيطرية بالعباسية

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** الهيئة القومية للرقابة والبحوث الدوائية

تهدف دراسة تأثير مستخلصات النباتات المختلفة على الفيروسات لعمل إستراتيجية جديدة و متطورة لعلاج هذه الأمراض الفيروسية . فلقد ثبت أن العديد من النباتات التجارية الطبية المختلفة لديها نشاط مضاد للفيروسات قوى وبالفعل تستخدم العديد من تلك المستخلصات فى علاج العديد من الأمراض المعدية التى قد تصيب الأنسان والحيوان. وتشمل الدراسة الحالية تقييم النشاط المضاد لفيروس روتا الأبقار الخاص بمستخلصات نبات الجوافة ونبات المرمريه . حيث أجريت الدراسة على خلايا الزرع النسيجى والفئران ولقد ثبت من هذه الدراسة أن مستخلص نبات الجوافة عند تركيزات تتراوح بين٠,٠٣ الى ٠,٦ ميكروجرام لكل مللى لديه تأثير مضاد لفيروس روتا الأبقار قوى حيث أن نصف الجرعة المعدية له أصغر من ٠,٠٣ ميكروجرام لكل مللى , بينما لم يثبت أن مستخلص نبات المرمريه عند تركيزات ما بين ٠,٠٣ الى ٠,٦ ميكروجرام لكل مللى له تأثير مضاد لفيروس روتا الأبقار.

يرجع التأثير المضاد لفيروس روتا الأبقار الخاص بمستخلص نبات الجوافة الى إعاقة تكاثر الفيروس فى أطواره المعديه المبكرة ولكن هذا التأثير قد يكون ضغيفا فى الأطوار المعديه المتأخرة بعد إختراق الفيروس للخلايا وعليه نوصى بإستخدام مستخلص نبات الجوافة كمادة مضادة فى لتجنب الإصابة بفيروس روتا الأبقار وبخاصة فى الحيوانات المعرضة للإصابة بالفيروس أو يوصى بإستخدام مستخلص الجوافة فى علاج المرض فى حالة الإصابة المبكرة.