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Assessment of a Probable Existence of an Antiviral Influence of a Phyto-Product (*Salvia Officinalis*) on Peste Des Petits Ruminants Attenuated Virus

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Abstract

Aqueous extract from leaves of sage (*Salvia officinalis*) was examined for its probable antiviral effect against Peste des Petits Ruminants attenuated virus (PPRV) using VERO cells. The cytotoxicity studies revealed that all the used dilutions of the aqueous extract of sage leaves were non-toxic to VERO cells. Incubation of the virus with the aqueous sage extract at concentration of 310.9µg/ml for 4 hours at 37°C showed complete reduction in the virus titer. Intracellular treatment of the VERO cells before infection with the PPR virus with different concentrations of the aqueous sage leaves extract revealed only one log reduction in the virus titer compared to the virus control used. However, the antiviral effect of the aqueous sage leaves extract for the virus-infected VERO cells showed complete reduction in the virus titer at concentration of 310.9 µg/ml while at concentration of 77.72 µg/ml there was only one log reduction ofthe virus titer compared to the virus control used. Aqueous sage leaves extract is recommended to be used safely during outbreaks of PPR virus in sheep and goats after studying its antiviral effect in sheep and goats, safety and side effects.

Keywords: Sage leaves; PPRV; Antiviral

Introduction

Peste des petits ruminants (PPR) (Gargadennec; Lalanne 1942) is an acute contagious viral disease of small ruminants caused by a morbillivirus in the family paramyxoviridae (Gibbs et al., 1979). The disease is characterized by fever, oculonasal discharges, stomatitis,

diarrhea and pneumonia. Peste des petits ruminants virus (PPRV) is transmitted by aerosols between animals living in close contact (Lefevre and Diallo, 1990). The disease occurs in most African countries from north Africa to Tanzania (Kwiatek et al., 2011) and in nearly all Middle Eastern countries to Turkey (Ozkul et al., 2002). PPR also widespread in countries from central Asia to South and South-East Africa (Banyard et al., 2010). Control of PPR is ensured via using of the live attenuated PPR virus vaccine, which is now widely commercially available (OIE, 2012). Peste des petits ruminant (PPR) is a highly contagious viral respiratory disease of small ruminants (Ogunsanmi et al., 2003; OIE 2008). It is one of the major impediments to improving productivity of small ruminants in the regions where it is endemic (Chaven et al., 2009). The disease has a seasonal variation with outbreaks occurring mainly in the early part of the rainy and cold seasons (CIDRAP, 2003). No specific measures are available to treat the disease but antibiotics were used to prevent secondary infection. Control of PPR outbreaks relies on movement control (quarantine) combined with focused (ring) vaccination and prophylactic immunization in high risk population (Radostitis et al., 2007). In a particular flock, the risk of outbreaks is greatly increased when a new stock is introduced or when animals are returned unsold from livestock market (Fentahun and Woldie, 2012).

*Salvia*is an important genus consisting of about 900 species in the family *Lamiaceae*. Many species of *Salvia* have been used in traditional herbal medicine worldwide. In one traditional herbal medicine, sage is used for many ailments including inflammation of the mouth and throat (Tucakov 1996).Extracts of different *Salvia* species have been examined for a number of biological activities including antimicrobial, anti-inflammatory and antioxidant activity (Ren et al., 2004). Tada et al., (1994) observed a potent antiviral activity against vesicular stomatitis virus (VSV) in crude extracts of *S. Officinalis* and compared the antiviral diterpenessafficinolid and sageon. Safficinolid induced reduction of VSV, while sageon exhibited virus inactivation activity against VSV and Herpes Simplex virus type 1 (HSV-1). Also, aqueous extract from sage leaves display potent anti-HIV-1 activity by increasing the virion density as described by (Geuenich et al., 2008).

This study aims to evaluate the antiviral effect of aqueous sage leaves extract against Peste des petits ruminants (PPR) virus *in vitro*.

Material and methods

1] Peste des Petits Ruminants virus (PPRV)

Peste des Petits Ruminants virus (PPRV) attenuated Nigerian 75/1 strain (Diallo et al., 1989) obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. It has a titer of7.0 $Log_{10}TCID_{50}$ / ml.

2] African Green Monkey Kidney cell line (VERO)

It was used for detection of the antiviral and cytotoxic effect of sage aqueous extract, VERO cells (Yasumura and Kawatika, 1963) were grown in culture medium consisting of minimal essential medium (MEM) supplemented with (penicillin-G sodium and streptomycin sulphate at final concentration of 100 IU/ml and 100 µg/ml, respectively). The medium was enriched with 10% newborn calf serum for cell growth. This proportion of serum was reduced to 2% for maintenance medium when the cell monolayers were completed (OIE, 2000).

3] Preparation of the aqueous sage leaves extract:

Dried sage leaves (*Salvia officinalis* L.) were identified microscopically and chromatographically according to their monographs in European Pharmacopeia. Aqueous extract was prepared by adding 100 ml of boiling distilled water to 10 grams of the dried leaves, and incubated for 15 min., subsequently filtered and cooled down. The resulting extract was sterile filtered, aliquoted and stored at -20°C according to Nolkemper et al., (2006). The aqueous extract was freeze-dried.

4] Determination of the cytotoxicity of Sage leaves aqueous extract on VERO cells:

VERO cells were seeded in 96–well micro-titer plates then incubated over night at 37°C. Two-fold serial dilutions of the aqueous sage leaves extract in fresh medium containing 2% newborn calf serum was added to the confluent cell monolayer and incubated for 7 days. Cytotoxicity was determined by examining cellular morphology after cell staining with crystal violet stain (Freshney, 1983; Doyle et al., 1995).

5] Extracellular treatment of the PPR virus:

Equal amounts of the 100 $TCID_{50}$ / ml of the PPR virus and 1/10, 1/20 and 1/40 of the aqueous extract dilutions were mixed together and incubated at 37 °C for 4 hours then serial tenfold dilutions were done from each mixture on previously seeded monolayer sheets of VERO cells in the

presence of virus control (not treated with the sage) and cell control (cells without addition of the virus) wells (Boseila and Abou Hatab, 2011). When the virus control wells showed CPE, the drop in the virus titer was calculated regarding to the titer of the positive virus control according to (DraganaŠmidling et al., 2008).

6] Intracellular treatment effect of the sage leaves extract against infection with PPR virus:

Two- fold serial dilutions of the aqueous sage leaves extract were inoculated into previously seeded VERO cell monolayers. After 90 min, cell cultures were rinsed with PBS and a virus suspension (100 $TCID_{50}$/ml) was added to each well of the treated cell monolayer (100 µl/ well). Cell control and virus control wells were involved in the test. The incubation was done for 6-7 days at 37°C till the presence of CPE in the virus control wells. Back titration was done to evaluate the antiviral activity of the aqueous sage leaves extract against PPR virus according to Dragana Šmidling et al., (2008).

7] Evaluation of the antiviral activity of the aqueous sage leaves extract:

A virus suspension (100 $TCID_{50}$/ml) was pre-incubated with VERO cells monolayer. After 90 min, cell cultures were rinsed with PBS and two-fold serial dilutions of the aqueous sage leaves extract were added to the infected cell monolayer. Cell control and virus control wells were involved in the test. The incubation was done for 6-7 days at 37°C till the presence of CPE in virus infectivity control wells. Back titration was done to evaluate the antiviral activity of the aqueous sage leaves extract against PPR virus according to Dragana Šmidling et al., (2008).

Results

Data presented in Table 1 showed that the aqueous sage leaves extract was safe (non-toxic) to VERO cells when used with different concentrations. As shown in Table 2, our results revealed that there was complete reduction in the titer of the virus when it was treated with 1/20 dilution of the aqueous sage leaves extract (310.9 µg/ml) while there were no log reduction in the virus titer when it was treated either with 1/40 dilution (155.45 µg/ml) or 1/80 dilution (77.72 µg/ml) of the aqueous sage leaves extract before infecting VERO cells.

Table 1.Cytotoxicity of the aqueous sage leaves extract on VERO cells

Dilution of the sage leaves extract	Concentration of sage leaves extract (µg / ml)	Cytotoxicity %
1/20	310.9	0 (Non-toxic)
1/40	155.45	0 (Non-toxic)
1/80	77.72	0 (Non-toxic)

Table 2. Extracellular treatment of the virus

Dilution of the sage leaves extract	Concentration of sage leaves extract (µg / ml)	The virus titer ($Log_{10}TCID_{50}$/ml)	The positive control virus titer ($Log_{10}TCID_{50}$/ml)
1/20	310.9	0.0	7.0
1/40	155.45	7.0	
1/80	77.72	7.0	

The figures depicted in Table 3 denoted that all the used concentrations of the aqueous sage leaves extract showed protection of the VERO cells before infection with PPR virus by only one log reduction compared to the virus control. There was no relationship between the amount of the active ingredients used in the extract and the reduction in the virus titer in case of intracellular protection of VERO cells before getting infected.

Table 3. Intracellular treatment of the cell line before infection with the virus

Dilution of the sage leaves extract	Concentration of sage leaves extract (µg / ml)	The virus titer ($Log_{10}TCID_{50}$/ml)	The positive control virus titer ($Log_{10}TCID_{50}$/ml)
1/20	310.90	6.0	7.0
1/40	155.45	6.0	
1/80	77.72	6.0	

Table (4) showed that the aqueous sage leaves extract at dilution of 1/20 (310.9 µg/ml) had antiviral effect on the infected cells (previously infected with the virus) and showed complete inhibition of the virus replication while by increasing the dilution of the extract the yield of the virus begins to increase again due to the decrease in the amount of the active ingredients found in the aqueous extract of sage.

Table 4. Antiviral evaluation of the virus infected cell line

Dilution of the sage leaves extract	Concentration of sage leaves extract (µg / ml)	The virus titer ($Log_{10}TCID_{50}$/ml)	The positive control virus titer ($Log_{10}TCID_{50}$/ml)
1/20	310.90	0.0	7.0
1/40	155.45	5.0	
1/80	77.72	6.0	

Discussion

Attempts to use antiviral drugs against PPR virus was continued as in case of the successful use of Riomycin against PPR virus for infected wadgs (Nwakundu et al., 2013).In this study we tried to find natural antiviral herbs that could be used simply in the field to treat infected animals to reduce the infections and spread of the infection in the flock to minimize the amount of the virus shedding in the field.

According to Vanden Berghe et al., (1986) when screening plant extract for antiviral activity *in vitro* one is looking for non-specific action of antiviral agents on infected cells. Concerning the results in the Table (1) it has been showed clearly that the aqueous sage leaves extract was safe (non-toxic) to VERO cells when used with different concentrations without showing any signs of apoptosis or change in cell morphology and these results agree with Geuenich et al., (2008) who found that aqueous sage leaves extract did not or only at very high concentrations interfere with cell viability. Also these results agree with Lima et al., (2007) who showed that aqueous and methanolic extracts of sage can protect significantly HepG2 cells against cell death when co-incubated with toxicant.

Regarding the result in Table 2, it was noticed that there was complete reduction in the titer of the virus when it was treated with 1/20 dilution of the aqueous sage leaves extract (310.9 µg/ml) while there were no log reduction in the virus titer when it was treated either with 1/40 dilution (155.45 µg/ml) or 1/80 dilution (77.72 µg/ml) of the aqueous sage leaves extract before infecting VERO cells. This result agree with that obtained by Geuenich et al., (2008) who stated that the aqueous *Lamiaceae* extract (sage) exhibited virucidal effect against enveloped viruses but not against the non-enveloped adenovirus type 5.

With regard to Table (3) when using the aqueous sage leaves extract with different concentrations before infecting VERO cells with the PPR virus, all the used concentrations of the aqueous sage leaves extract showed only one log reduction compared with the virus control. There was no relationship between the amount of the active ingredients used in the extract and the reduction in the virus titer in case of intracellular protection of VERO cells before getting infection but in Table (4) when the same concentrations of the extract were used after infection of the VERO cells with the PPR virus, the results showed that the aqueous sage leaves extract at dilution of 1/20 (310.9 µg/ml) had antiviral effect on the infected cells (previously infected with the virus) and showed complete inhibition of the virus replication, while by increasing the dilution of the extract the yield of the virus begins to increase again due to the decrease in the amount of the active ingredients found in the aqueous extract of sage representing direct relationship between the amount of the active ingredients and the antiviral effect on the virus. These results agree with results obtained by Geuenich et al., (2008) they stated that the aqueous *Lamiaceae* extract exhibited virucidal effect against enveloped viruses. Also these resultsagree with those showed in Boseila and Abou Hatab (2011).Using different concentrations of aqueous sage leaves extract against FMD virus, they found that it had antiviral activity against FMD virus at concentration of 155.45 µg /ml when used for infected cells (after virus infection).

Refering to Baricevic and Bartol (2000) potent extracts of crude aerial parts of sage *(S. officinalis)* were displayed by two abietanediterpenoids. One of them was safficinolide, which was active against VSV (vesicular stomatitis virus), while the other was sageone that showed virus inactivation activity against Herpes Simplex virus type 1 (Tada et al., 1994). According to Bulgarian researchers, water and alcoholic extracts of sage were active against influenza, herpes simplex and vaccinia viruses (Manolova et al., 1995). This preparation was officially approved for clinical use in Bulgaria. Schnitzler et al., (2008) stated that the aqueous extracts of sage contain oligomers of caffeic acid derivatives that were identified to have antiviral activity. Moreover, the observed virucidal activity of sage (*Lamiaceae*) was considered to be due to phenolics such as flavonoids, tannins and caffeic acid derivatives which were reported previously to inactivate herpes simplex viruses by blocking ligands or

receptors on the surface of viruses and host cells, respectively as stated by Kaul et al., (1985); Vanden Berghe et al., (1986); Jassim and Naji (2003).

Conclusion

Aqueous sage leaves extract could be used safely as a good antiviral against PPR virus in sheep and goats during outbreaks. However, sage extract may be used for the infected animals to diminish the virus shedding in the field and control the disease in Egypt. Further studies should be done to identify the active principles responsible for the antiviral activity of the sage leaves in animals.

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استبيان وجود تأثير محتمل لمستخلص نباتى (مريمية) مضاد لفيروس طاعون المجترات الصغيرة المستضعف

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لقد أوضحت الدراسة الحالية أن المستخلص المائى من نبات الساج (المرمية) ذو تأثير مضاد لفيروس طاعون المجترات الصغيرة المستضعف كما تبين ذلك من اختفاء التأثير الممرض للفيروس على خلايا الزرع النسيجى.

وبناءا على دراسة التغيرات الخلوية للمستخلص المائى لنبات المريمية على خلايا الزرع النسيجى (كلى القرد الأفريقى الأخضر) أن المستخلص آمن الاستخدام بجميع تركيزاته المستخدمه حيث أنه لم يسبب أى تغيرات فى شكل و درجة نمو الخلايا.

كما أوضحت الدراسة أن معالجة الفيروس بالمستخلص المائى لنبات الساج (المرمية)قبل الحقن على الخلايا أدى ذلك الى حماية كاملة للخلايا عند تركيز 310.9ميكروجرامو لكن عند تركيز 155.45 ميكروجرام لم يكن له أى تأثير على عيارية الفيروس فى حين أن معالجة الخلايا بتركيزات مختلفة من المستخلص المائى لنبات الساج (المرمية) قبل العدوى بالفيروس يخفض عيارية الفيروس بوحدة عيارية واحدة. أما معالجة الخلايا بعد العدوى بالفيروس فقد أدى ذلك الى حماية كاملة للخلايا عند تركيز 310.9ميكروجرام و لكن عند تركيز 77.72 ميكروجرام فخفضت عيارية الفيروس بمقدار وحدة عيارية فقط.

والأمر يحتاج الى مزيد من الدراسة لامكانيةاستخدام المستخلص المائى لنبات الساج (المرمية) أثناء انتشار الأوبئةلمنع العدوى بفيروس طاعون المجترات الصغيرة و ذلك بعد دراسة تأثيره فى الحيوانات.