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# ANTIVIRAL ACTIVITY *IN VITRO* OF FIVE SELECTED INDIGENOUS PLANTS AGAINST BOVINE HERPES VIRUS-1 (BHV-1) AND BOVINE VIRAL DIARRHOEA VIRUS (BVDV)

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## ABSTRACT

*In this study, we investigated the in vitro antiviral activity of five selected plant extracts against Bovine Herpes Virus-1 (BHV-1) subtype 1.1 and Bovine Viral Diarrhoea Virus (BVDV) genotype 1, which with other viral and bacterial agents cause Bovine Respiratory Disease Complex (BRDC). Ethanol (alcohol) and aqueous extracts were prepared for all five plant species studied. Two prepared samples of aqueous plant extracts/macerates had antiviral effect against BHV-1 as a representative of herpesvirus (DNA virus), but none of the samples showed an antiviral effect against BVDV genotype 1, a NADL strain, that belongs to RNA viruses. In our study, Selectivity Index ($SI = CTC_{50}/EC_{50}$) for aqueous extracts of Matricaria chamomilla ($SI = 32$) and Achillea millefolium ($SI = \geq 8$) indicates a significant selective inhibition of DNA virus (BHV-1, strain TN41). On the basis of the obtained results, future research is needed, which would reveal the active ingredients of the investigated plants and mechanisms of their antiviral effect.*

***Key words*** *- antiviral activity, BHV-1, BVDV genotype 1, indigenous plants*

## INTRODUCTION

The reasons for restrictions on the use of antiviral drugs in human and veterinary medicine in comparison with the use of antimicrobial agents are reflected in viral mutation, new viruses, toxic effects, the severity of viral diseases, ability of virus to survive intracellularly, the high costs and the non-availability of specific antiviral chemical agents against veterinary pathogens. Deficiency of effective antiviral drugs require discoveries a new effective antiviral compounds. Most antiviral against viruses of veterinary importance are still used as animal models in the development of human antiviral drugs (e.g BVDV like surrogate model for the hepatitis C virus). Plants are naturally gifted at the synthesis of medicinal compounds, and allow creation of new, cheap drugs with high therapeutic potential. Today insists on the advancement of analytical chemistry tools, the development of standardization and extraction methods, and the standardization of virus assays (Pushpa et al., 2013; Zeedan, 2016). Herbs are rich source of phytochemicals with different biological activities including antiviral effects. Plants contain various compounds that inhibit different parts of the replication cycles of various types of DNA and RNA viruses. This antiviral activity is generally attributed to polyphenols, rosmarinic acid, chlorogenic and caffeic acid (Jassim and Naji, 2003), which interact with each other, altering the effect of a particular active compound (Gobbo-Neto and Lopes, 2007).

BHV-1 is a member of the subfamily *Alphaherpesvirinae*. Because of its importance in veterinary medicine, BHV-1 is listed on list B disease by the office International des Epizooties (OIE) (Jones, 2003). BHV-1 is the cause of many different diseases (rhinotracheitis, vulvovaginitis, balanoposthitis, abortions, conjunctivitis and generalized systemic infections) and important cofactor in the BRDC, which responsible for major economic losses in both beef and dairy production (Jones and Chowdhury, 2010; Biswas et al., 2013; Kurćubić et al., 2018). Khan et al. (2005) reported that medicinal plants are an important tools of controlling viral diseases caused by herpesviruses in both humans and animals. A large number of studies in which plant extracts were used have shown *in vitro* anti-BHV-1 activities (del Barrio and Parra, 2000; Simoni et al., 2007; Melo et al., 2008; Gupta et al., 2010; Pilau et al., 2011; Barros et al., 2012; Fernandes et al., 2012; Abdallah et al., 2013). According to our knowledge of the available literature data, antiviral activities of five different herb extracts examined in our study against BHV-1 and BVDV have never been tested.

BVDV is a non-arthropod-borne member pestivirus within the *Flavirida*e family, and causes infections of domestic and wild ruminants

worldwide (Lindenbach et al., 2007). The presence or absence of visible CPE in infected cell cultures conditioned the division of BVDV into cytopathic (CP) or non-cytopathic (NCP) biotypes (Baker, 1995). Based on antigenic and genetic properties, two species of the BVDV can be distinguished, BVDV-1 and BVDV-2. Recent data (Ridpath et al., 2010) indicate that it has been identified 12 BVDV-1 subtypes (BVDV-1a - BVDV-1l) and 2 BVDV-2 subtypes (BVDV-2a and BVDV-2b). BVDV infections causes respiratory and reproductive problems: transplacental infection and fetal death, congenital malformations, neonatal and postnatal mortality, mucosal diseases, retarded growth and poor performance of surviving animals (Bolin et al., 1985; Roeder and Harkness, 1986). Also, BVDV is one of the most problematic viral agents involved in the development of BRDC. Antiviral properties of plant extracts against BVDV have been tested, and different levels of activity have been proven (Koseki et al., 1990; Ruffa et al., 2004; Pilau et al., 2011).

A commonly accepted protocol for testing of antivirus sensitivity is not available (Swierkosz and Hodinka, 1999; Khan et al., 2005). To investigate antiviral activity, the most commonly used methods are fewest different techniques based on cytopathic effect or cell viability (Swierkosz and Hodinka, 1999; Khan et al., 2005; Da Silva et al., 2006; Astani et al., 2010). Tetrazolium dye (MTT) colorimetric assay (Khan et al., 2005), measures cell viability and it has been applied to access antiviral activity against different viruses (Freitas et al., 2009). Sylvester (2011) reported that the MTT assay is rapid, convenient, and economical, and become a very popular technique for quantification of viable cells in culture.

The main objective of this study is to determine the existence and severity of the virucidal effect (anti-viral activity) of the extracts of 5 various plants obtained by 2 different extraction methods (ethanol and aqueous extracts) as well as checking viability of viral particles. The first aim in this study is to determine the concentration of plant extracts that cause cytotoxicity to 50% of the cells (the so-called 50% cytotoxic concentration - $CTC_{50}$). Also, the cytotoxicity of extracts of different plants obtained by different methods of extraction, as well as various solvents, was determined on the cell cultures that we later used in the experiment.

## MATERIALS AND METHODS

*Plant materials:*

A total of 5 indigenous plants were collected from different regions of the Central Serbia, as presented in Table 1.

Table 1. List of indigenous plants (n=5) screened in the present study

| No. | Plant name | Family |
|---|---|---|
| 1. | *Anchusa officinalis* L. | Boraginaceae |
| 2. | *Althaea officinalis* | Malvaceae |
| 3. | *Halacsya sendtneri* | Boraginaceae |
| 4. | *Matricaria chamomilla* | Asteraceae |
| 5. | *Achillea millefolium* | Asteraceae |

*Preparation of extracts - Extraction by process of maceration (MAC):*

The maceration is a one-extraction chopped prescribed drugs, prescribed solvent at room temperature. Plant samples (10.0 g) were extracted by 300 mL 96% ethanol for ethanol extracts or water for aqueous extracts as a solvents in laboratory conditions at a temperature of 22 ºC in a sheltered, dry place for seven days with occasional shaking. After seven days extract was filtered through filter paper (Whatman, No.1), then concentrated to dry mass by a rotary evaporator (Devarot, Elektromedicina, Ljubljana, Slovenia) under vacuum and dried at 60 ºC to constant weight. The dried extracts were stored in a dark glass bottle at 4 ºC to prevent oxidative damage.

Ethanol (alcohol) and aqueous extracts were prepared for all five plant species studied. In the presentation of the results (Table 2), the plant ethanol extracts (EE) are numbered from 1 to 5, and aqueous plant extracts (AE) are marked with numbers 1a to 5a.

*Cell lines and Viruses:*

The Madin-Darby Bovine Kidney (MDBK) cell line (ATCC, CCL-22) was used in the experiment. The cell line was grown in Eagle minimum essential medium (Eagle MEM) Hepes modification (Sigma-Aldrich, USA) supplemented with 10 % fetal bovine serum (FBS) (Capricorn, Germany), 80 µg/mL gentamicin and 100 µg/mL benzyl penicillin.

For estimation of antiviral activity of tested plant extracts, the animal pathogenic viruses Bovine herpesvirus-1 (BHV-1; IBR/IPV virus) strain TN41 (American Bioresearch, USA) as representative of DNA, and Bovine Viral Diarrhoea Virus (BVDV) strain NADL (NVSL, Ames, USA) as

representative of RNA viruses, were used in the experiment. Both virus strains were growth and titrated on MDBK cell line.

To determine the titer of virus stocks, serial ten-fold dilutions were prepared in Eagle MEM medium followed by inoculation in MDBK cell monolayers contained in 96-well microtiter plates using 100 μL/well. Seven wells were used per dilution. Inoculated cells were incubated at 37 °C in up to 72 hours. The highest dilution showing CPE was considered as the end point. Virus titers were calculated as $TCID_{50}$/mL by the protocol of Reed and Muench (1938).

*Cytotoxicity assay*

The plant extracts obtained as water and alcohol extracts were assayed for cell toxicity prior to testing in antiviral studies. Cytotoxicity measurements were based on alteration of normal cell morphology (Benencia and Courreges, 1999; Yucharoen et al., 2011). The extracts were reconstituted by two-fold dilution with Eagle MEM Hepes modification (Sigma-Aldrich, USA) with 8% of FBS (Capricorn, Germany) and antibiotics. Serial two-fold dilutions of the plant extracts preparations ranging from concentrations from 9.77 to 1250 μg/mL were added in triplicate wells of 96-well tissue culture plate (Sarstedt, Germany) with confluent MDBK cell monolayers formed after 24h of cell seeding. Treated cultures were incubated for a 72h at 37 $^{o}$C. The cultures were observed daily for evidence of cytopathic effect (partial or complete loss of the monolayer, or rounding or shrinkage of the cells). The dose, causing 50% cytopathic effect (CPE) with respect to cell control (intact cells), was estimated (50% cytotoxic concentration - $CTC_{50}$) and calculated according to protocol of Reed and Muench (1938).

*Antiviral activity screening:*

Different nontoxic concentrations (lower than $CTC_{50}$) of plant extracts were tested for antiviral property by cytopathic effect (CPE) inhibition assay against 100 $TCID_{50}$ virus concentrations of BHV-1 and BVDV viruses similarly as it was described by Vijayan et al. (2004). Briefly, the MDBK cells were seeded in a 96-well microtiter plate with 30.000 cells per well, incubated at 37 ºC in incubator for a period of 24h. The two-fold dilutions of plant extracts in Eagle MEM ranging from 5 mg/mL to 39.06 μg/mL were prepared in another microtiter plate. For each dilution of plant extracts six wells in microtiter plate were used. In another microtiter plate wells the prepared dilutions of tested samples in amount of 100 μL were

mixed with 100 µL (100 $TCID_{50}$) of BHV-1, as well as BVDV virus in triplicate and incubated at 37 ºC for 2 hours. After incubation, the plates with MDBK cell monolayer were washed with fresh Eagle MEM and inoculated with 100 µL of prepared extracts dilutions/virus mixtures. In addition, 100 µL of Eagle MEM with 8% of FBS and antibiotics was added to each well. The final concentrations of each tested plant extract in microtiter plates were from 1250 to 9.77 µg/mL. In each microtiter plate the positive controls (MDBK cells inoculated with 100 $TCID_{50}$ of BHV-1, as well as with 100 $TCID_{50}$ of BVDV), and negative controls (only MDBK cells) in triplicate were set-up. The reactions were incubated at 37 ºC for three days. Every 24h the observation was made and cytopathic effects were recorded. Anti-BHV-1 and anti-BVDV activity was determined by the inhibition of cytopathic effect compared with control (untreated cells infected with 100 $TCID_{50}$ of BHV-1 or BVDV viruses). This inhibition of CPE, i.e. protection of the cells from the virus activity, directly connected to the antiviral activity of tested plant extracts, was scored. The concentration reducing CPE by 50% (50% effective concentration - $EC_{50}$) with respect to virus control was estimated. The selectivity index (SI) was determined from the ratio $CTC_{50}/EC_{50}$. SI ≥ 4 was considered to stand for a significant selective inhibition (Sokmen et al., 2004).

## RESULTS AND DISCUSSION

The evaluation of the antiviral activity indicated that two of the aqueous tested plant extracts (4a and 5a - *Matricaria chamomilla* and *Achillea millefolium*) inhibited the replication of BHV-1 in MDBK cell line (Table 2). It should be noted that all of the plant extracts samples were toxic to the MDBK cell line used, especially in higher concentration that possibly could exhibit virucidal effect. The water extracts were less toxic than alcohol (ethanol) extracts.

In our study, selectivity index (SI = $CTC_{50}/EC_{50}$) for herb extracts 4a (SI = 32) and 5a (SI = ≥ 8) indicates a significant selective inhibition of DNA virus (BHV-1, strain TN41). Sokmen et al. (2004) state that significant selective inhibition of Herpes simplex virus type 1 (HSV-1) if the SI is ≥ 4. In this study, MeOH extract of herbal parts of *Origanum acutidens* have SI = 36.0, and MeOH extract from callus cultures of *O. acutidens* have SI = 15.4. In a study by Boubaker-Elandalousi et al. (2014) with non-cytotoxic *Thymus capitata* extracts in the prevention of Bovine herpesvirus-1 infection in cell cultures, the selectivity index for essential oil (EO) was 14.49, for ethanol extract 3.95 and for aqueous extract 1.81. High cytotoxic value and a low inhibitory concentration gave the highest selectivity index for EO, indicating

that it is the most effective antiviral extract. Parreira et al. (2017) in his *in vitro* evaluation of extracts from leaves of *Drimys brasiliensis* (Winteraceae) against BHV-1 (Los Angeles strain - LA) exhibited strong antiviral activity.

Table 2. Cytotoxicity and antiviral effects of tested plant extracts

| | | | Virus inhibitory effect | | | |
|---|---|---|---|---|---|---|
| No. of sample | | Cytotoxicity | BHV-1 (IBR/IPV) strain TN41 | | BVDV strain NADL | |
| | | $CTC_{50}$ (µg/mL)[a] | $EC_{50}$ (µg/mL)[b] | SI[c] | $EC_{50}$ (µg/mL)[b] | SI[c] |
| 1 | EE | ≥ 312.5 | > 312.5 | < CTC[d] | > 312.5 | < CTC |
| 1a | AE | > 1250.0 | ≥ 312.5 | 4 | > 1250.0 | ND[d] |
| 2 | EE | ≥ 156.3 | > 156.3 | < CTC | > 156.3 | < CTC |
| 2a | AE | > 1250.0 | > 1250.0 | ND | > 1250.0 | ND |
| 3 | EE | ≥ 312.5 | > 312.5 | < CTC | > 312.5 | < CTC |
| 3a | AE | > 1250.0 | ≥ 1250.0 | ≥ 1 | > 1250.0 | ND |
| 4 | EE | ≥ 312.5 | > 312.5 | < CTC | > 312.5 | < CTC |
| 4a | AE | > 1250.0 | ≥ 39.06 | ≥ 32 | > 1250.0 | ND |
| 5 | EE | ≥ 156.3 | ≥ 156.3 | < CTC | > 156.3 | < CTC |
| 5a | AE | > 1250.0 | ≥ 156.3 | ≥ 8 | > 1250.0 | < CTC |

[a] Fifty percent cytotoxic concentration - $CTC_{50}$
[b] Fifty percent effective concentration - $EC_{50}$
[c] Selectivity index ($CTC_{50}/EC_{50}$)
[d] antiviral activity is lower that cytotoxic concentration (< CTE) or not detected (ND)
EE - ethanol extracts
AE - aqueous plant extracts

Chiang et al. (2002) demonstrated an antiviral effect of the soluble phenolic compounds of *Plantago major*, caffeic acid and chlorogenic acid, against herpesvirus, but McCutcheon et al. (1995) reported that the methanol and ethanol extracts of *P. major* tested against herpesvirus did not exhibit antiviral activity. Variations in antiviral activity from these extracts are a consequence of differences in the structure of the viral envelope, which alters the interaction between the viruses and herb compounds.

Pilau et al. (2011) performed the MTT test (3-4,5-dimethythiazol-2yl)-2,5-diphenyl tetrazolium bromide) to determine the selectivity index (SI) of the essential oil against human and animal viruses, which was equal to 13.1, 7.4, 10.8, 9.7, and 7.2 for acyclovir resistant herpes simplex virus type 1 (ACVR-HHV-1), acyclovir-sensitive HHV-1, human respiratory syncytial virus (HRSV), bovine herpesvirus type 2 (BoHV-2), and bovine viral diarrhoea virus (BVDV), respectively. The human rotavirus (RV) and bovine herpesvirus type 1 (BoHV-1) and 5 were not inhibited by the essential oil.

Fifteen Argentine medicinal plants were tested for their antiviral activity *in vitro* against bovine viral diarrhoea virus type 1 (BVDV-1), NADL strain, ATCC VR 534. The Selective Index of the active extract ($SI_{extract} = CC_{50extract}/EC_{50\ extract}$) of *Coronopus didymus* ($SI_{extract}$=110.7), *Juglans australis* ($SI_{extract}$=8.1) and *Lippia alba* ($SI_{extract}$=19.2) against BVDV-1 justify a further analysis. The determined $SI_{extract}$ from the three plants active against BVDV-1 are higher that other eight examined plants (Ruffa et al., 2004).

Sometimes, certain compounds of a crude aqueous extract could be modified by temperature and the period of incubation during extraction, and thus altering the antiviral activity. When the crude aqueous extract of *Amaranthus spinosus* was heated for 30 min at 50-60 °C the antiviral activity against BVD was lost (Koseki et al., 1990). The result of this work may also be an explanation for the absence of BVDV antiviral activity as demonstrated in our study - antiviral activity is lower that cytotoxic concentration (< CTE) or not detected (ND).

## CONCLUSIONS

The present study reports for the first time the antiviral activity of five examined herb macerates/extracts. The conducted tests and the obtained results definitely indicate the existence of differences in the virucidal effect of macerates of different plants, as well as in relation to the virus against which the antiviral effect has been tested. Namely, research has found that the antiviral effect, and without the accompanying toxic effect on cell culture as living tissue, was detected for aqueous macerate/extracts compared to ethanol macerates/extracts. Ethanol as a carrier/medium in which dilution and extraction was carried out has caused a cytotoxic effect, and probably also a virucidal but is the result of the effect of alcohol rather than extract/macerate of plants. Two prepared samples of aqueous plant extracts/macerates had antiviral effect against BHV-1 as a representative of herpesvirus (DNA virus), but none of the samples showed an antiviral effect

against BVDV genotype 1, a NADL strain, that belongs to RNA viruses. In our study, selectivity index (SI = $CTC_{50}/EC_{50}$) for aqueous extracts of *Matricaria chamomilla* (SI = 32) and *Achillea millefolium* (SI = $\geq$ 8) indicates a significant selective inhibition of DNA virus (BHV-1, strain TN41).

We believe that it is necessary to improve the investigation of the use of herb macerate/extracts as antiviral agents in the future, and apply new approaches, such as using a low dose of synergistic antiviral combination of herb macerate/extracts. It would also be necessary to examine the mechanism of action of biologically active substances that are isolated and characterized from the examined plant macerates/extracts. The above mentioned study was carried out *in vitro* and for future research it is imperative that they be carried out on an animal model *in vivo*.

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