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Evaluation of Antiviral Activity of Selected Anise Oil as An Essential Oil Against Bovine Herpes Virus Type -1 *In vitro*

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Abstract: In this study, the antiviral activity of anise oil against bovine herpes virus type-1 (BHV-1) was investigated in the cell culture. Anise oil was found to be non-toxic to MDBK cells up to concentration 100µg/ml and inhibited the growth and development of BHV-1 in a dose-dependent manner in MDBK cells. In order to study the possible mechanism of the antiviral activity of anise oil, MDBK cells were treated with anise oil as a pre, simultaneous and post viral infection treatment assay. At maximum non-cytotoxic concentrations of anise oil, the inhibition of virus yield showed that the treated cells with anise oil for 1 hour after infection can significantly reduce the virus titer from 10^6 to 10^3 $TCID_{50}$/ml but the inhibitory effect of anise oil was not significant in other cases. Although the precise mechanism has not yet to be defined, our work indicated that anise oil could inhibit growth and development of BHV-1 in *in vitro* cultured cells.

Key words: Bovine Herpes Virus Type-1 (BHV-1) · Anise oil · Antiviral activity · MDBK cell · $TCID_{50.}$

INTRODUCTION

Bovine herpes virus type-1 (BHV-1) are DNA viruses belonging to the family herpesviridae and are responsible for a variety of mild to severe disease which are sometimes life threatening [1], BHV-1 has a great importance in veterinary medicine as it classified in a list B disease by the office International des Epizooties (OIE) [2].

In Egypt, since 1960s attention was drawn to BHV-1 as one of the most significant causes of great economic losses in feedlot and dairy farms; mainly due to deaths from pneumoentritis, abortion and prolonged feeding periods programs [3]. A very effective treatment for BHV-1 is available since the introduction of acyclovir in the 1970s and it is the most commonly used chemotherapy [4]. Antiviral agents might produce toxic side effects. In addition, the emergence of virus strains resistant to commonly used anti herpes virus drugs [5]. The development of viral resistance toward antiviral agents enhances the need for new effective compounds against viral infection. In the recent years, there has been an increasing interest in the use of natural products and some questions concerning the safety of synthetic compounds have encouraged more detailed studies of plant resources. Various essential oils and their components posses pharmacological effects, demonstrating anti inflammatory, antioxidant and anti cancerogenic properties [6]. Medicinal plants produce a variety of chemical constituents with the potential to inhibit viral replication and compounds from natural sources are of interest as possible sources to control viral infection. The anti herpes activity of several essential oils of different plant sources as well as some constituents of essential oils had been demonstrated previously [7]. The application of anise oil as an essential oil met the standard demands of current pharmacopeias [8]. This essential oil is rich in trans-anethole and consist of about 80% of this phenyl propanoid [9]. Phenyl propanoids present in these essential oils contribute to their antiviral activity [10]. Therefore, the present study was undertaken for the evaluation of antiviral activity of selected anise oil as an essential oil against bovine herpes virus type-1.

MATERIALS AND METHODS

Anise Oil: Anise oil was purchased from (Sigma-Aldrich). Anise oil was dissolved in ethanol and was

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further diluted in medium for cell culture experiments in different concentrations as 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 µg /ml [11].

BHV-1 and Cell Culture: BHV-1 was kindly obtained from Virology Department, Faculty of Veterinary Medicine, Zagazig University. Continuous cell line of Madin Darby Bovine Kidney (MDBK) cells were purchased from VACSERA Company, Agoza, Giza, Egypt. MDBK cells were grown in monolayer culture with Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin and amphotericin B (0.025µg/ml) and maintained at 37°C with 5% CO2 for 24 hours for confluency. MDBK cells were cultured onto 96- well and 6 -well culture plates for cytotoxicity and antiviral assays, respectively and propagated in monolayer culture with Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal calf serum at 37°C in an atmosphere of 5% of CO_2 [12]. Stock virus was propagated and titrated and stored at -80°C until used as previously described [13]. Virus titer was obtained by limit dilution method and expressed as 50% tissue culture infectious dose per ml ($TCID_{50}$/ml) [14]. The infection titer of the stock virus solution was $10^6 TCID_{50}$/ ml.

Cell Viability Test: For cytotoxicity assays, MDBK cells were seeded onto 96- well plates and incubated for 24 hours at 37°C. The medium was removed and fresh Dulbecco's modified Eagle's medium (DMEM) containing the appropriate dilutions of anise oil were added onto subconfluent cells in three replicates for each concentration of anise oil. Wells containing medium without anise oil were also included on each plate as controls. Cell destruction was evaluated during microscopic examination. Cytoxicity was expressed as the 50% cytotoxic concentration (CC_{50}), that is, the concentration of anise oil required to reduce viral cytopathogenicity by 50% (within micro tray well) [15].

Virus Yields Inhibition Assay: The maximum non cytotoxic concentration of anise oil was always used to evaluate the mode of antiviral action. In order to study the possible mechanism of the antiviral activity of anise oil, MDBK cells were treated with anise oil as a pre, simultaneous and post BHV-1 infection. Cell monolayers were pretreated with anise oil prior to inoculation with BHV-1 by adding the oil to medium followed by incubation for 1 hour at 37°C before viral infection. For determination of antiviral activity of the anise oil at simultaneous and post BHV-1 infection; the anise oil was added together with the virus and after 1, 4, 8 and 12 hour post viral infections at 37°C, respectively as typically performed in antiviral susceptibility studies. Each assay was run in three replicates. Wells containing medium with 1% ethanol but no anise oil was also included on each plate as controls. Virus titer was determined by the endpoint dilution method and expressed as $TCID_{50}$ /ml by comparison with virus control [16].

RESULTS

Cell Viability Test: The assessment of cytotoicity was performed with anise oil serially diluted in DMEM with different concentrations as 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 µg/ml. The maximum non-cytotoxic concentration of anise oil was about 100µg/ml and this concentration did not cause any change in MDBK cells and was used in further experiments in this work. However, cell lysis and degeneration were observed with increasing intensity at higher concentrations of the anise oil (Figure 1).

Virus Yields Inhibition Assay: The potent antiviral activity of anise oil was determined against BHV-1 *in vitro.* The inhibition of virus yield by the anise oil was evaluated by $TCID_{50}$/ml assay in MDBK cells. When host cells were pretreated with anise oil prior to infection, none of the tested oils showed significant effects on viral infection as well as simultaneous infection treatment assay (Figure 2). On the other hand at postinfection treatment assay, the infectivity of BHV-1was inhibited especially at 1 hour post viral infection, where the virus titer of the supernatant dropped from 10^6 to 10^3 $TCID_{50}$/ml. The inhibitory effect of anise oil was not significant at 4, 8 and 12 hour post infection treatment assay where BHV-1 replicated normally and the viral titers recovered from supernatant of infected cultures were ranged from $10^{5.5}$- $10^{5.9}$ $TCID_{50}$/ ml as virus control.

DISCUSSION

Despite the successes in the treatment of some virus diseases during the past three decades, the search for new antiviral drugs remains an area of active investigation [4]. Effective treatment is not available for many viral infections. Moreover, the selection of resistant and cross-resistant mutants caused partially by the narrow

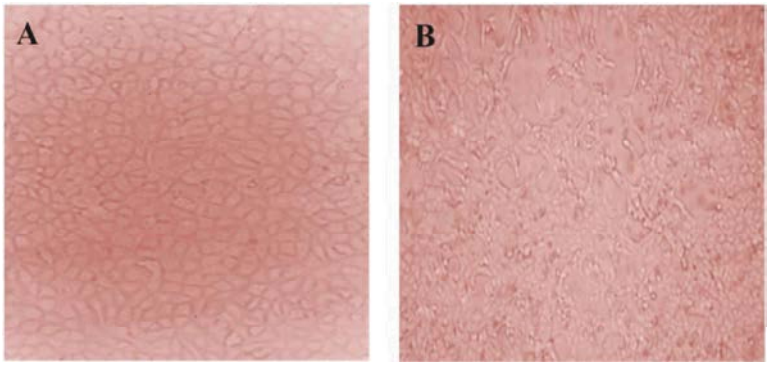

Fig. 1: Cytotoxicity effect of Anise oil on MDBK cells (X100). A-Normal MDBK cell cultures showing a confluent monolayer sheet of cells. B-MDBK cells treated with high concentrations of anise showing cell rounding and clumping

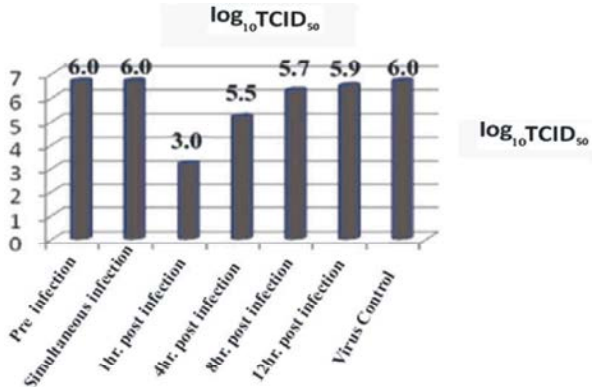

Fig. 2: Antiviral activity of the anise oil (pre, simultaneous and post BHV-1 infection treatment assay) on virus yield in MDBK cells

spectrum of the mechanism of action, as well as potential toxic side-effects demand the discovery of new drugs [5]. To study the antiviral activity of natural extracts, as well as synthetic drugs or derivatives of known antiviral drugs, rapid, highly standardized and inexpensive screening procedures are needed for clinically important viruses. Several essential oils have been proposed as promising alternative therapeutic tools [15]. The present study was initiated to evaluate the inhibitory effect of the anise oil against bovine herpes virus type -1 (BHV-1). In this study, the effect of different concentrations of the anise oil on MDBK cells was determined after 3 days of incubation by cell viability test. The anise oil had no cytotoxicity on MDBK cells up to a concentration of 100 µg/ml. In order to determine the mode of the inhibitory effect, anise oil was added at different stages during the viral infection cycle at maximum non cytotoxic concentrations of the anise oil. The inhibition of virus yield showed that treating the cells with the anise oil 1 hour after infection can significantly reduce the virus titer from 10^6 to 10^3 $TCID_{50}$/ml. The inhibitory effect of the anise oil was not significant in other cases. The essential oil directly inactivates herpesvirus and might interfere with virion envelope structures or mask viral structures that are necessary for adsorption or entry into host cells [17], which is in accordance with our results and other essential oils [18]. These results need further studies to know the mechanism of action of essential oils using suitable animal models.

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