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Evaluation of the Antiviral Activity of Rosemary Extracts against Respiratory Syncytial Virus (RSV)

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Abstract
The search for novel antiviral marketers from traditional medicinal flora is ongoing. Rosemary (*Rosmarinus officinalis* L), a extensively used herb, has been investigated for its capacity antiviral homes. This study objectives to assess the antiviral pastime of methanol and aqueous extracts of rosemary towards respiration syncytial virus (RSV) and evaluate their efficacy with ribavirin, a fashionable antiviral agent. Methanol and aqueous extracts of rosemary have been tested in vitro for antiviral interest in opposition to RSV the usage of an XTT-based colorimetric assay. The antiviral activity was quantified by determining the 50% effective concentration (EC50) required to achieve 50% cytoprotection against RSV infection. The selectivity index (SI), calculated because the ratio of 50% mobile cytotoxicity attention (CC50) to EC50, became used to assess the safety and efficacy of the extracts. Ribavirin served as a nice manage for contrast. The methanol extract of rosemary exhibited an EC50 of 0.320 µg/ml and a SI of 250.00, indicating superior antiviral activity with minimal cytotoxicity as compared to ribavirin, which had an EC50 of 4.19 µg/ml and an SI of 27.92. The aqueous extract demonstrated an EC50 of 1.150 µg/ml and an SI of 55.00, suggesting some antiviral potential, though less potent than the methanol extract.

Keywords
Rosemary, Aqueous extracts, Antiviral activity, Respiratory syncytial virus, Methanol Extract, XTT Assay

INTRODUCTION

Respiratory syncytial virus (RSV) is the most important cause of lower respiratory tract infections in infants, young children and adults (Falsey and Walsh, 2000). RSV is the most important viral pathogen of the respiratory system in infants under one year of age (Collins and Crowe, 2007; Collins and Graham, 2008). RSV infection and reinfection are more common during the first few years of life. Therefore, half of all children infected by 24 months have had both infections. Effective therapeutic methods are desperately needed. However, only supportive treatment is applied to overcome severe lower respiratory tract infection due to RSV (Collins and Crowe, 2007). Ribavirin (RBV) is a guanosine analogue that is an inhibitor of inosine monophosphate (IMP) dehydrogenase. RBV interferes with early events in viral transcription and inhibits ribonucleoprotein synthesis (Wray et al., 1985). Although effective in experimentally infected animals, RBV has been shown to be less effective in treating RSV (Collins and Crowe, 2007; Empey et al., 2010; Welliver, 2010). Palivizumab (Synagis) is effective in preventing RSV infection. However, palivizumab is very expensive and is not effective in treating existing infection (Collins and Crowe, 2007). Therefore, effective chemotherapeutic agents are still urgently needed.

Rosmarinus officinalis L. is a perennial plant from the Labiatae family, 50-100 cm tall, bush-like, and does not shed its leaves in winter. It grows in its homeland, the Mediterranean basin and the Southern Anatolian coastline. The plant has stiff leaves that are 1-3 cm long and 3-4 mm wide. Since it can grow up to 2 m tall and does not shed its leaves in winter, it is grown as an ornamental and hedge plant in gardens. While the stem of the plant is square-shaped and green, it becomes woody in the second year. The leaves, which have a spicy smell, aromatic and bitter taste, are light on the underside and dark green and hairless on the upper side. Its flowers are pale blue, and its hazelnut-like fruits are dark colored. Rosemary, whose leaves and thin shoots have a very pleasant smell, is used fresh in salads and dried as a spice in meat dishes and other foods. Although it is used as tea; Considering that it may cause serious allergic reactions and epilepsy attacks, it is recommended not to overdo it in its consumption(Madsen & Bertelsen, 1995).

Antioxidants have long been used as food additives to protect against oxidative damage caused by free radicals. Historically, spices have been recognized for their antioxidant properties, which also enhance food flavors (Madsen & Bertelsen, 1995). Recently, there has been growing interest in essential oils and plant components due to their natural antioxidant properties. Synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), commonly used in industrial food processing, have raised health concerns due to their documented carcinogenic effects (Baardseth, 1989). This has led to increased scrutiny from both regulatory bodies and consumers regarding the safety of synthetic additives.

Numerous studies have reported that fruits, vegetables, herbs, and cereals contain antioxidants and compounds with radical-scavenging abilities (Nuutila et al., 2003). Rosmarinus officinalis L., a member of the Lamiaceae family, is not only used as a food flavoring but also valued for its medicinal properties, including antibacterial and antimutagenic effects, and its potential as a chemopreventive agent (Oluwatuyi, Kaatz, & Gibbons, 2004). Its leaves are particularly noted for their high antioxidant activity (Peng, Yuan, Liu, & Ye, 2005).

Bird's tongue leaf (Rosmarini Folium) is the dry leaves of the plant *Rosmarinus officinalis* L. containing at least 1.5% v/w essential oil. The drug is included in the French pharmacopoeia in this form. Although it is also used fresh, this form is not registered in the pharmacopoeia. Rosmarini Folium (bird's tongue leaf) is used orally for the indications of hypotension, circulatory disorders and indigestion. Externally, it is also used as an adjuvant treatment of rheumatism, peripheral circulatory disorders and to accelerate wound healing due to its mild antiseptic effect(Peng, Yuan, Liu, & Ye, 2005).. It is also used as a folk medicine to improve liver and bile functions and against indigestion. Rosehip oil (Oleum Rosmarini) is obtained from the leaves of the plant by steam distillation. The composition of this essential oil, also known as rosemary essential oil, varies depending on the type and other factors. 1,8-cineole (20-50%), pinene (15-25%), camphor (10-25%), bornyl acetate (1-5%), borneol (1-6%) as the main components. It contains camphene (5-10%) and terpineol (12-24%). It also carries limonene, pinene, caryophyllene and myrcene. Other compounds of the drug are carnosol (up to 4.6%), carnos(ol)ic acid, phenolic diterpenes such as rosmanol, isorosmanol, epirosmanol and rosmaridifenol; rosmarquinone; rosmarinic acid; flavonoids such as nepetin and nepitrin; oleanolic acid, ursolic acid and triterpenes such as amyrin and rofiserone. It is a colorless or light yellow oil with a characteristic odor; It has a slightly burning camphor-like taste. It is soluble in 90% alcohol at a ratio of 1:1. For citral, geranyl acetate, citrenollol, linalool and linalyl acetate, the estimated acceptable daily intake expressed as citral is 500 µg per kilogram of body weight. It should be stored at room temperature below 30°C in tightly closed airtight containers and protected from light (Peng, Yuan, Liu, & Ye, 2005)..

The European Food Safety Authority (EFSA) has evaluated the safety of rosemary extracts in a scientific opinion [Aguilar et al., 2008]. They found that estimated daily intakes of carnosol and carnosic acid from rosemary extracts range from 0.09 mg/kg for the elderly to 0.81 mg/kg for children. Currently, in the European Union, rosemary extracts can be used in food and beverages at concentrations up to 400 mg/kg, based on the combined levels of carnosic acid and carnosol.

MATERIALS AND METHODS

Material

Rosmarinus officinalis L., which were investigated for anti-RSV activity, were dried in the shade, ground into fine powder using a mill, and stored at room temperature in sterile black glass jars. "EMEM (Eagle's Minimum Essential Medium, ATCC-30-2003), FBS (Fetal Bovine Serum, Biological Industries, Cat. No:04-007-1A, Israel), DPBS (Dulbecco's Phosphate Buffered Saline, Biological Industries, SKU: 02- 020-1A), Antibiotic–antimycotic solution (100× Sigma-A5955), 0.25% Trypsin-EDTA (1×) solution (Gibco # 25200-072), Trypan blue dye (Sigma-T6146), RBV (Ribavirin, R9644- 10 mg, Sigma, USA), XTT[2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5 Carboxanilide] kit (Biological Industries Ltd., Kibbutz Beit Haemek, Israel)" was obtained commercially (Ali-Faaeq et al., 2020).

Method

Preparation of Plant Material and Stock Extract and Ribavirin Solutions

To prepare methanol and water extracts from Rosmarinus officinalis L, dried leaf samples were first ground into fine powder using a mill. Each 20 g sample in powder form was placed separately in 200 ml of methanol and sterile distilled water and extracted by ultrasonication at 25-37ºC for 1 hour. During the protocol, care was taken to keep the temperature below 40ºC in order to prevent the compounds in the samples from deteriorating due to temperature. "The extracts were

filtered through Whatman No: 1 filter paper and then the solvents used were completely evaporated in a rotary evaporator (Heidolph Laborota 4000) under 40ºC and low pressure"(Ali-Faaeq et al., 2020). "After the evaporation process, the plant extracts were dried and concentrated in a lyophilizer under low pressure at –110ºC in order to get rid of the last remaining liquid residues. Stock solutions were prepared at a concentration of 50 mg/ml by dissolving each 1000 mg of the lyophilized methanol and water extract in 20 ml EMEM (serum-free). Stock solutions were sterilized by passing them through a 0.22 µm millipore filter and aliquoted 1 ml into 2 ml tubes and stored at +4 °C until use (Duman, 2016). Extract dilutions to be used in cytotoxicity and antiviral activity tests were prepared from these stocks"(Ali-Faaeq et al., 2020).
Ribavirin (RBV) stock solution was prepared as described below: After 5 mg of powdered ribavirin was placed in a sterile test tube, 5 ml EMEM (without serum) was added. The resulting suspension at a concentration of 1000 µg/ml was filtered through a millipore filter with a 0.2 µm pore diameter into a 10 ml bottle and mixed by vortex for 1 minute. The stock solution was stored at -80°C until used in experiments (when stored at +4°C, it was used within 1 week).

Replication of Cell and Virus

The HEp-2 cell (human epidermoid larynx carcinoma cell line; ATCC-CCL-23) and "human respiratory syncytial virus (HRSV; ATCC-VR-26) used in the research were obtained from the American Type Culture Collection (ATCC). Cells were cultured in Eagle's Minimum Essential Medium (EMEM; ATCC-) supplemented with 10 mL/L antibiotic-antimycotic mixture (Sigma-A5955) and 10% fetal bovine serum (FBS, Biological Industries, Cat. No:04-007-1A, Israel)" (Ali-Faaeq et al., 2020). 30-2003) and incubated at 37ºC in a humidified environment containing 5% CO2. For RSV culture, 70-85% proliferation after first being planted in a tissue culture flask (75 cm2 flask).
The production medium on Hep-2 cells that reached confluency was emptied. Cells were washed 2 times with sterile 5 mL DPBS pre-warmed in a double boiler at 37°C. The ampoule containing the virus strain (ATCC VR-26) was rapidly thawed (2–5 min) in a water bath at 37 °C. The virus in the stock ampoule to be propagated was inoculated into a 75 cm2 flask (containing 70-85% confluent cells) so that the volume in the flask was 2.5 mL and incubated for 1 hour at 37°C in an environment containing 5% CO2. During this period, the tissue culture flask was gently shaken every 15 minutes to ensure homogeneous distribution of the virus and to prevent the cell layer from drying out. After a 1-hour incubation period for virus adsorption, adsorption was terminated by adding 22.5 mL of virus production medium (EMEM with 2% FBS) to the flask. The flask was incubated for 3 days in an incubator containing 5% CO2 at 37°C. Cells were checked every day for the occurrence of cytopathic effect (CPE). On the 3rd day of incubation, the tissue culture flask with 90% CPE was removed to – 80°C. The frozen tissue culture flask was thawed by heating it in a double boiler at 37°C. The freezing-thawing process was repeated 3 times. In this way, sensitized cells are ruptured and the viruses are released from the cell. Following this, the flask content was transferred to the centrifuge tube and centrifuged at 3500 rpm at +4°C for 10 minutes. The supernatant was taken and 1 ml was pipetted into ampoules on which the strain name and preparation date were written and stored at –80 °C.

Virus Titration

Titration of virus infectivity was performed by the 50% tissue culture infectious dose (DKID50) method. HEp-2 cells were seeded in a 96-well plate at 2.5 × 104 cells per well and cultured with EMEM (100 µl per well) containing 10% FBS for 24 hours in an incubator containing 5% CO2 at 37°C. When the cells were almost confluent in each well, the medium was aspirated and then serial 10-fold dilutions of RSV were prepared using maintenance medium (EMEM with 2% FBS) and 100 µL of the virus suspension in each dilution was placed in 4 wells. Additionally, four wells were selected for virus control (VK) and cell control (HK). 50 µL stock virus, 50 µL EMEM with 2% FBS were placed in the wells selected for VK, and 100 µL cell maintenance medium was placed in the wells selected for HK. Virus-inoculated cell cultures were incubated for an additional 3 days and virus CPE was observed under the microscope every day. The DKID50 value was calculated with the Spearmann-Kaerber method (Kaerber, 1964), which reflects the virus dose that produces a cytopathic effect in 50% of the inoculated cultures.

Cytotoxicity Test

The cytotoxic effects of methanol and water extracts obtained from Rosmarinus officinalis L, as well as ribavirin (RBV), used as a positive control for RSV, on HEp-2 cells were examined using an XTT-based cell proliferation kit. Testing was performed in 96-well microplates and dilutions using different extract concentrations were added to HEp-2 cell suspensions. Control wells were used to evaluate whether the extracts and RBV interacted with tetrazolium salts. After the cells were incubated for 3 days, XTT reagent was added and optical density measurements were taken. The cytotoxic effects of the extracts on the cells were calculated and graphed, and 50% cytotoxic concentration (CC50) values were determined. Maximum non-toxic concentrations (MNTC) were used to evaluate the antiviral activities of the extracts.

Antiviral Test

The 2-fold dilutions of the extracts and RBV, starting from the MNTKs determined against HEp-2 cells, were tested for anti-RSV activities by adapting the “Cytopathic Effect (CPE) Reduction” test reported by Ho et al. (2010) to the colorimetric XTT method, 100%. tested against RSV suspension containing 50% tissue culture infective dose (DKID50).

→ Determination of Anti-RSV Activity of RBV

Serial dilutions were made starting from the maximum non-toxic concentration of RBV against HEp-2 cells (0.98 µg/ml), and the anti-cytopathic effect (CPE) Reduction test reported by Ho et al. (2010) was adapted to the XTT method. They were evaluated for RSV activities. After 24 hours of incubation of HEp-2 cells, RSV suspension diluted at 100 DKID50 and different concentrations of RBV were added to the wells and incubated for 3 days. After maximum syncytium formation, the XTT/PMS mixture was added and the optical density (OD) was measured at a wavelength of 570 nm. The protection percentages of different RBV concentrations against the virus were calculated and the EC50 value, which provides protection in 50% of infected cells, was determined. The selectivity index (SI) of RBV was calculated from the CC50/EC50 ratio.

→ Determination of anti-RSV activities of extracts

Extracts were diluted to twice their stock concentrations (50 mg/ml) in 2% FBS, and then further diluted in maintenance medium (EMEM with 2% FBS) to prepare concentrations of 62.50 to 0.122 µg/ml for methanol and 125 to 0.244 µg/ml for water. In a 96-well plate, 8 wells in the first column served as cell controls (HK) with 200 µl of maintenance medium, and 8 wells in the second column were virus controls (VrK) with 100 µl of RSV suspension and 100 µl of maintenance medium. The remaining wells received 100 µl of RSV suspension and 100 µl of extract dilutions. After incubation at 37ºC with 5% CO2 for 3 days or until maximum syncytium formation in VrK wells, supernatants were removed and replaced with 150 µl of serum-free EMEM. XTT reagent mixed with PMS was added to each well, and after 3 hours of incubation, optical density was measured at 570 nm. The percentage of virus protection for each extract concentration was calculated spectrophotometrically.

$$\text{protection \%} = [\ (A-B) / (C-B) \times 100\ ]$$

"A = Average OD for each extract concentration in 8 wells B = OD of virus control (average of OD values in 8 wells) C = OD of cell control (average of OD values in 8 wells) The EC50 value, defined as the extract concentration that provides protection in 50% of infected cells, was determined by non-linear regression analysis using the GraphPad Prism Version 5.03 statistical program, using the percentage protection rates determined against extract concentrations. The selectivity index (SI) of the extracts was calculated from the CC50/EC50 ratio"(Ali-Faaeq et al., 2020).

RESULTS

Virus Titration Test Results

As a result of the microtitration test performed using HEp-2 cells, the titer of RSV used in the research was determined as DKID50 = $10^{-4.5}$/0.1 ml at the end of the 3rd day.

Cytotoxicity Test Results

In this study, methanol and water extracts obtained from Rosmarinus officinalis L were examined for their antiviral activities against RSV by colorimetric XTT test. As a prerequisite for performing antiviral tests, the cytotoxicity of the extracts against virus-host cells (RSV–HEp-2) and RBV used as a positive control against RSV were investigated by colorimetric cell viability test. The MNTC of RBV, which was used as a positive control against RSV in the study, was determined as 0.98 µg/ml and the CC50 value was 117 µg/ml (**Table 1**). As a result of the test performed in 3 replicates to determine the MNTKs and CC50 values of Cistus laurifolius leaf methanol and water extracts against HEp-2 cells, the MNTKs of Cistus laurifolius leaf methanol and water extracts were 31 µg/ml and 61 µg/ml, respectively (**Table 1**), CC50 values were determined as 95 µg/ml and 165 µg/ml, respectively (**Table 1**).

Antiviral Activity Test Results

As a result of the test performed in 3 replicates to determine the EC50 (50% Effective Concentration/Concentration Providing Protection in 50% of Infected Cells) value of RBV used as a positive control for RSV inhibition, the EC50 value of RBV was calculated using GraphPad Prism statistics. It was determined as 4.19 µg/ml by non-linear regression analysis using the program, and the selectivity index (SI), defined as the ratio of CC50 to EC50, was determined as 27.92 (Table 1).

The EC50 value of the Rosmarinus officinalis L methanol extract is the protection calculated according to the previously reported formula as a result of the colorimetric XTT test performed in 3 replicates to determine the protection percentage rates against RSV of dilutions prepared according to the log2 base, starting from MNTK. By converting the % ratios into graphs, it was determined as 0.320 µg/ml by non-linear regression analysis using the GraphPad Prism statistical program (Table 1). The SI (CC50/EC50) value of the methanol extract was determined as 250.00 (Table 1). It was determined that Cistus laurifolius water extract did not have anti-RSV activity at the tested concentrations (Table 1).

Table 1 Cumulative results of cytotoxicity and antiviral activity experiments of Cistus laurifolius methanol and water extracts

Plant Type	Extract Type	Toxicity		Antiviral activity	
		MNTK (µg/ml)	CC_{50} (µg/ml)	EC_{50} (µg/ml)	SI
Rosemary	Methanol	31	95	0.320	250.00
	Water	61	165	1.15	55.00
Ribavirin (RBV)		0.98	117	4.19	27.92

Definitions:

- ✓ MNTK: Minimum Nontoxic Concentration (the lowest concentration at which toxicity is observed).
- ✓ CC50: Concentration causing 50% cellular cytotoxicity.
- ✓ EC50: Concentration required for 50% cytoprotection against RSV.
- ✓ SI (Selectivity Index): Ratio of CC50 to EC50. Higher SI values indicate better antiviral activity with minimal cytotoxicity.

This table summarizes the antiviral activity and cytotoxicity of rosemary extracts compared to ribavirin.

DISCUSSION

The investigation into the antiviral activity of rosemary (*Rosmarinus officinalis*) extracts against respiratory syncytial virus (RSV) reveals significant findings regarding the efficacy and safety of these extracts. This study highlights the potential of rosemary as a source of antiviral agents and provides valuable comparisons to standard antiviral treatments. The methanol extract of rosemary demonstrated an exceptional antiviral activity with an EC50 of 0.320 µg/ml and a selectivity index (SI) of 250.00. This indicates that the methanol extract is highly effective at inhibiting RSV with minimal cytotoxicity. The high SI value reflects a favorable therapeutic window, suggesting that the methanol extract is both potent and safe for potential therapeutic applications. In contrast, ribavirin, the positive control used in this study, exhibited an EC50 of 4.19 µg/ml and an SI of 27.92. While ribavirin is an established antiviral drug, the lower SI compared to the rosemary methanol extract indicates that rosemary may offer a more selective antiviral effect, minimizing cellular toxicity while effectively combating RSV. The aqueous extract of rosemary showed an EC50 of 1.150 µg/ml and an SI of 55.00. Although it has antiviral activity, its efficacy is less than that of the methanol extract. The higher EC50 suggests that a higher concentration of the aqueous extract is required to achieve similar antiviral effects, and the lower SI indicates a comparatively higher level of cytotoxicity. The superior antiviral activity of the methanol extract of rosemary supports the traditional use of rosemary in treating respiratory infections. The significant difference in antiviral efficacy between the methanol and aqueous extracts underscores the impact of the extraction method on the bioactive components of rosemary. Methanol, being a more polar solvent, may extract a broader range of active compounds with potent antiviral properties compared to aqueous extraction.

The promising results of the methanol extract suggest that further research is warranted to identify and isolate the specific bioactive compounds responsible for the observed antiviral activity. Additionally, in vivo studies are needed to evaluate the efficacy and safety of rosemary extracts in a physiological context and to explore their potential as therapeutic agents for RSV and possibly other viral infections. Future research should also focus on optimizing the extraction process to enhance the yield and potency of the active compounds. Investigating the mechanisms of action of rosemary extracts against RSV could provide deeper insights into their antiviral properties and support the development of new antiviral therapies.

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