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## Bioactivity of Rosemary and Sage Against Measles

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### Abstract

Two methanolic extracts of common medicinal plants; *Rosmarinus officinalis* (rosemary), *Salvia triloba* (sage), were tested for their antiviral activity against Measles (MV) virus in vitro with the aim of evaluation of the biological activity (cytotoxicity on measles virus) of these aromatic, traditionally used plants in Jordan. A colorimetric tetrazolium-based (MTT) assay as well as visual evaluation of cell morphology using inverted light microscopy has been applied to test cytotoxicity of the different plants concentrations. Antiviral properties of the plant extracts were determined by cytopathic effect inhibition assay using african monkey kidney (Vero) cells. Cytotoxicity results showed that rosemary and sage extracts were toxic at the concentrations 50 and 100 µg/ml to Vero cells. The % inhibition of the Measles virus infectivity was evaluated, Interestingly sage showed a dose dependant inhibition of Measles virus cytopathic effect (CPE) at all virus dilutions A good correlation at the second dilution of the virus was achieved with 50% effective concentration (EC<sub>50</sub> =14.74 µg/ml) While rosemary extract exhibited dose-dependent inhibition of Measles virus cytopathic effect at the first dilution of the virus only with an EC<sub>50</sub> of 23.96 µg/ml and a considerable inhibition of the viral infectivity with the lowest dilution of virus. Consequently rosemary and sage could be potentially promising for treatment of measles virus and could possess antiviral agents that may act against other viruses too.

**Keywords:** cytopathic effect; Cytotoxicity; *Rosmarinus officinalis*; *Salvia triloba*.

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## **1. Introduction**

The name Measles is derived from the Latin, *misellus*, meaning miserable. The disease is also sometimes known as *rubeola* (from *rubeolus*, Latin for reddish) or *morbilli* (from *morbus*, Latin for disease) [1].

Measles is considered one of the most serious common childhood diseases. It is estimated that Measles virus (MV) infects about 40 million children, and causes death of about 500,000 children worldwide each year [2]. Measles virus infects the body through the respiratory tract, where it causes a primary infection in the respiratory epithelium, then spreads to the immune cells, and to a wide range of endothelial and epithelial tissues throughout the body [3]. There is no specific treatment for Measles, and most patients with uncomplicated Measles can recover spontaneously with rest and symptomatic treatment [4].

Vaccination against Measles is the major intervention for combating the disease and limits its spread and complications. The vaccine is given as attenuated live virus, and as a part of a multiple vaccine includes Measles, Mumps, and Rubella (MMR) viruses. The vaccination has been introduced in developed countries, and it could substantially decrease the incidence of the disease [5].

Children are usually immunized against measles by the age of 18 months; the vaccine is not given earlier because children under that age usually retain immunoglobulins (antibodies) against the disease transmitted from their mothers during pregnancy. A booster dose is usually given at the age of 4 or 5. [6]. Although vaccination against Measles has markedly succeeded in controlling the disease and limiting its spread, it could not eliminate the disease due to the high infectivity of the virus, the relatively poor immunogenicity of the vaccine under the age of 9 months, and the difficulties in the distribution of the vaccine [7].

Despite the existence of effective live-attenuated vaccines, MV remains a serious threat to human health globally, where several live loss annually. Recent outbreaks have been attributed to declining herd immunity as a result of limited access to vaccination in some developed countries and reduced vaccination coverage due to parental concerns about vaccination safety. Taken together, these facts make desirable the development of novel therapeutics that could be produced cost-effectively and that could be used for the rapid control of local outbreaks and improved case management to limit severe outcomes of infection [4]. Moreover, vaccination programs could result in progressive weakness in the natural defense mechanisms since vaccine-induced immunity is less potent than the naturally acquired immunity [8].

In recent years the identification and evaluation of the potential benefits of natural compounds has become an important area of interest. *Rosmarinus officinalis* Lamiaceae (rosemary) and *Salvia triloba* Lamiaceae (sage) are a valuable aromatic plants that are increasingly being pursued as suitable alternative sources for discovery of a new and biologically active compounds and one of the attractive sources for discovery of antiviral agents.

*Rosmarinus officinalis* L. (Lamiaceae), more popularly known as “rosemary”, is an aromatic evergreen herb shrub that is widely distributed in the Mediterranean region and cultivated in Jordan. Locally it is called by *hasalban* or *iklil al- Jabal*. Rosemary is a greatly valued medicinal herb that is widely used in pharmaceutical products and traditional medicine. It is well known for its antioxidant [9], Anti-inflammatory [10]. Anticancer

activity against different types of cancer; ovarian cancer cell lines [11], cervical cancer [12] prostate cancer cells [13] and antispasmodic [14]. Antidepressant-like effect in animal models [15], anti-thrombotic effect [16] and it is used for obesity, constipation, kidney stones, hypertension, common cold, abdominal pains, ulcer, flatulence, toothache and edema [17].

*Salvia triloba* Lamiaceae is a small, perennial, evergreen sub shrub, with woody stems, grayish leaves, and blue to purplish flowers, more popularly known as “sage”. It is native to the Mediterranean region, mairamyah is the familiar name of sage that is known by the Jordanians.

*Salvia* essential oils have been reported to exhibit antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) [18]. *Salvia triloba* has cytotoxic effect against some cancer cell lines including human larynx epidermoid carcinoma (HEp-2) [19]. Beside that *S. triloba* exhibited antiproliferative activity against the adenocarcinoma of breast cell line (MCF7) [20]. Moreover, *salvia* possess a sedative and hypnotic effect [21].

The leaves of *S. triloba* are recommended for easing headaches, toothaches, common colds, and digestive problems. Finally it is used as gargle for oral infections and externally for wound healing [17] and as antispasmodic [22].

Taken together, the necessity of identifying new, safer, more efficacious and cost-effective therapeutic approaches to control Measles, markedly emerges. The plant kingdom especially aromatic plants and their essential oils have become a target for the search for new and biologically active compounds and one of the attractive sources for discovery of antiviral agents. Therefore, the present study aimed to investigate the antiviral activity of rosemary and sage that may have to be efficacious in preventing and treating measles diseases.

## **2. Materials and methods**

### ***2.1. Plant collection***

Leaves of rosemary and sage plants were collected; the plant material was dried at room temperature and milled into fine powder. Voucher specimen was identified by Dr. Tawaha K. from the department of pharmaceutical sciences of the Jordan University.

Plant material was extracted using 96% methanol. In brief, for each plant an alcoholic extract was prepared by weighing 100gm of powdered dried plant, then soaking in 500ml of methanol solution at room temperature to get a final concentration of 1gm/5ml. The plants were soaked for 7 days then filtered, after that the filtrate was evaporated to dryness using a rotary evaporator. Then, each extract was left in the incubator for 7 days to ensure dryness. Crude extracts obtained were used in different concentrations.

### ***2.2. Preparation of extracts***

Using electronic balance, 50mg of the plants extracts were weighed, dissolved in 50 ml of minimum essential media (MEM) with 2% heat-inactivated fetal bovine serum (FBS) to get a final concentration of 1 mg/ml which

was sterilized using a syringe filter paper with .2 $\mu$ m pore size. The sterile plant solution with the concentration of 1mg/ml was used as a stock solution for preparing further 6-fold dilutions with the concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 $\mu$ g/ml, to determine their cytotoxic concentrations and screening their antiviral activities.

### **2.3. Cell line and cell culture**

Vero cells were maintained in (MEM) supplemented with 10% (FBS), 50 $\mu$ g/ml of Gentamicin sulfate, 2mM of L-Glutamine, 2mM of Penicillin streptomycin , 0.01% of 2-Mercaptoethanol and 10mM of Hepes buffer system . The cells were sub cultured every 2-3 days at 37 °C in a humidified 5% CO<sub>2</sub> incubator. In cell toxicity and antiviral experiments, MEM supplemented with 2% FBS was used for performing assays.

All cells were propagated in a humidified 5% CO<sub>2</sub> incubator at 37°C. First, all cells in 75 cm<sup>2</sup> flasks were washed with 3–5 ml of phosphate buffer saline (PBS), then 1–2 ml of trypsin was added to each flask until the cells detached. An equal amount of fresh MEM was then added for each cell line with gentle pipetting was performed to disturb any clumps and ensure getting a uniform single cell suspension.

The frequency and ratio of the cell propagation was distinct for Vero cells were propagated every 2-3 days. Cells were counted using .4% trypan blue dye; where 100 $\mu$ l of the dye was mixed with 25  $\mu$ l of the harvested cells in a 0.5 ml epindorphe, then 100 $\mu$ l of stained cell suspension was transferred to edge of hemocytometer counting chamber and counted using light microscope.

### **2.4. Viruses**

Measles reference vaccine were used. The freeze-dried reference vaccine was reconstituted in 0.5 ml sterile water for injection. The starting concentrations of the Measles virus were 1000 Log CCID<sub>50</sub>, a serial eight-fold dilutions (10<sup>-1</sup>-10<sup>-4.5</sup> Log CCID<sub>50</sub> /ml) of the virus were prepared in MEM containing 2% FBS. Measles virus was grown in Vero cells and titrated by the method of cytopathic effect inhibition assay, cytopathic effect were observed daily using inverted microscope.

### **2.5. Cytotoxicity assay**

The plant extracts were assayed for cell toxicity before testing in antiviral studies. Cytotoxicity measurements were based on two parameters: (1) Alteration of normal cell Morphology using inverted microscope and (2) viability of the cells present in the culture using MTT assay. The cells were monitored daily for evidence of cytopathic effect (partial or complete loss of the monolayer, rounding or shrinkage of the cells, or a granular appearance in the cytoplasm) using inverted microscope.

### **2.6. Performing MTT assay**

Cells were seeded into 96-well plates at a density of 6 $\times$ 10<sup>3</sup> cells per well for Vero cells and incubated for three hours and half at 37 °C. Cells monolayer formed after 3hr of cell seeding were incubated with different

concentrations of plants extracts which were dissolved in MEM with 2% FBS and incubated for 7 days. At the 7th day, MTT assay was performed briefly, as follows: the medium was removed and cells in each well were incubated with 20 µl of MTT solution (5 mg/ml) for 4 h at 37°C. MTT solution was then discarded and 200µl dimethyl sulfoxide (DMSO) was added to dissolve insoluble formazan crystal. Optical density was measured at 570 nm and 630 nm using Eliza reader; Data were obtained from triplicate wells.

### **2.7. Antiviral activity assay**

The antiviral testing of the plants extracts was carried out by cytopathic effect reduction assay. In brief, 10 wells of microtitration plate, with Vero cells (6×10<sup>3</sup>) cells per well were inoculated with each dilution of Measles suspension to get eight dilutions of viruses with 10 replicates for each, after that serial four-fold dilutions of crude extracts were added simultaneously, and incubated for 1.5 hours at room temperature to allow adsorption of viruses.

As positive control, cells were infected with the same concentrations of virus but without the addition of plant extracts, and as a negative control, only MEM with 2%FBS was added to the cells. The plates were incubated at 37°C in a humidified CO<sub>2</sub> atmosphere for 7 days and visualized microscopically for any changes in cell appearance compared with normal control cells at the end of incubation period. The number of wells showed CPE were recorded on day 7 post infections and compared with the virus control. The reduction of virus multiplication was calculated as % of inhibition viral infectivity; which was calculated as follows

$$\% \text{ of inhibition} = 100 - \frac{\text{CPE of virus in the presence of plant extract}}{\text{CPE of virus control (with out plant)}} \times 100$$

The concentration reducing CPE by 50% in respect to virus control was estimated from graphic plots and was defined as 50% effective concentration (EC<sub>50</sub>).

## **3. Results**

### **3.1. Toxicity of extracts against cell lines**

Light micrographs showed that treatment of Vero cells with the highest concentrations of rosemary and sage extracts (50 and 100µg/ml) resulted in cell death; The cell monolayers were photographed at about 7days post treatment of an infected Vero cell monolayer and compared with untreated cells; which means that rosemary and sage extracts were toxic at these concentrations to Vero cells. While, at a concentration of 25µg/ml and below rosemary and sage extracts had no impact on either cell viability or proliferation of Vero cells.

### **3.2. Infectivity of the different dilutions of Measles virus**

The four dilutions (10<sup>-3</sup>-10<sup>-4.5</sup>) of the Measles virus; Measles did not show any activity (no cytopathic effects were observed in all wells contained cells and virus using inverted microscope).What meant that Measles virus became not infective any more at those dilutions because of the very low concentrations of it which had not the ability to infect the host cells (Vero Cells) and did not cause any CPE at all. So the antiviral activity of the plant

extracts could be evaluated and detected only at the first four dilutions ( $10^{-1}$  to  $10^{-2.5}$ ) of the virus.

### 3.3. Antiviral activity of the plant extracts as seen by inverted microscope

The two plant extracts, belonging to two different plant species out of two families, were studied in respect to their antiviral activity that was expected on the basis of CPE of the virus-infected confluent monolayer of Vero cells. The extent of cell damage was determined by the presence of CPE when compared to infected untreated cells (virus control) and uninfected untreated cells (cells control) by microscopic examination. The CPE caused by Measles virus on the Vero cell monolayer is characterized by one or more of the following features: (1) fusion of the Vero cells with one another (2) formation of syncytia (3) clearing of monolayer due to lysis of the cells.

### 3.4. Antiviral activity of the plant extracts using MTT assay

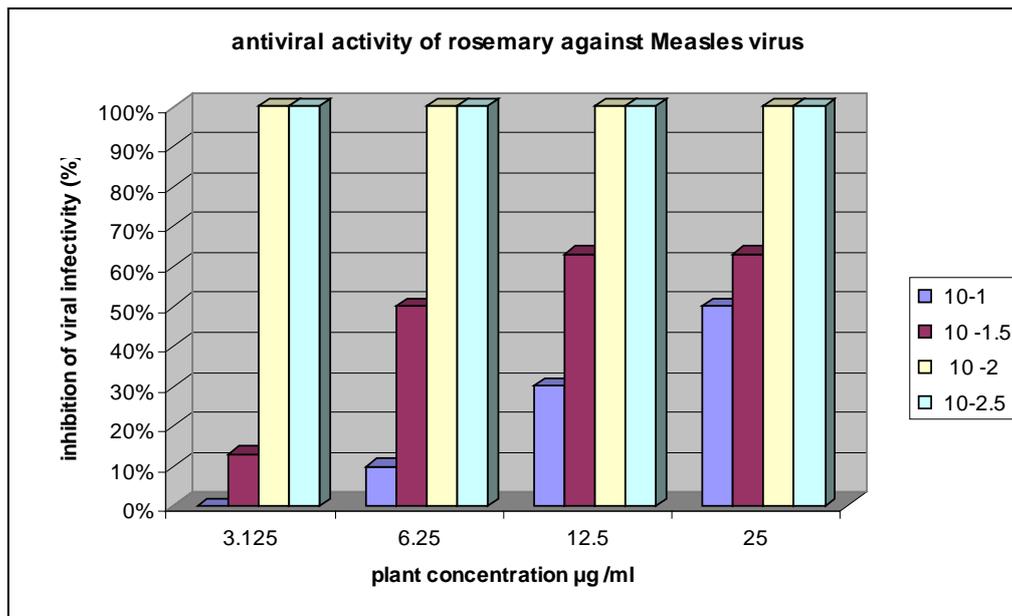
Rosemary extract exhibited dose-dependent inhibition of Measles virus cytopathic effect at the first dilution of virus as shown in Table 1 and Figure 1. The % of inhibition was reduced in treated cultures with a high correlation ( $r^2 = 0.967$ ) and an EC50 value of (23.96)  $\mu\text{g/ml}$  as shown in Figure 2. It was noticed that rosemary extract inhibited the viral infectivity considerably at the third and the fourth dilutions of the virus

**Table 1:** % Inhibition of Measles virus infectivity in the presence of rosemary extract at different dilutions of the virus

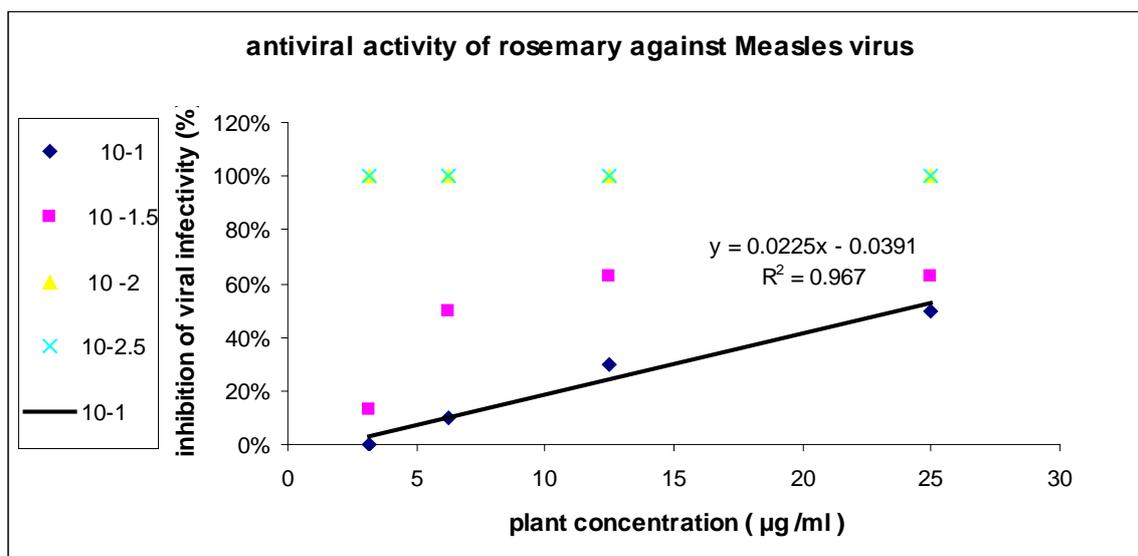
Plant concentration $\mu\text{g/ml}$	Virus dilutions			
	$10^{-1}$	$10^{-1.5}$	$10^{-2}$	$10^{-2.5}$
25	50%	63%	100%	100%
12.5	30%	63%	100%	100%
6.25	10%	50%	100%	100%
3.125	0%	13%	100%	100%

**Table 2:** % Inhibition of Measles virus infectivity in the presence of sage extract at different dilutions of virus

Plant concentration $\mu\text{g/ml}$	Virus dilutions			
	$10^{-1}$	$10^{-1.5}$	$10^{-2}$	$10^{-2.5}$
25	80%	88%	100%	100%
12.5	20%	50%	67%	100%
6.25	20%	13%	50%	100%
3.125	0%	0%	33%	100%

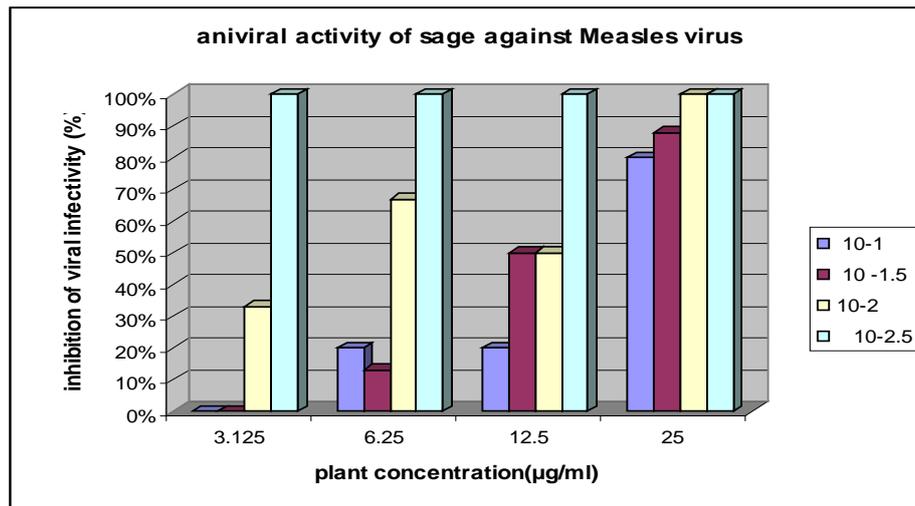


**Figure 1:** Antiviral activity: concentration-response curve of the inhibition of cytopathic effect of four fold-dilutions of measles virus by rosemary extract

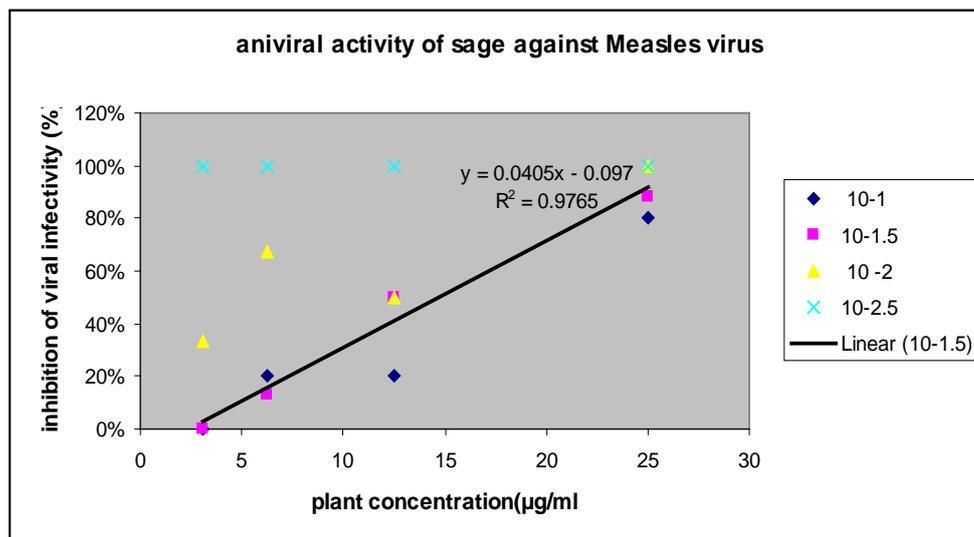


**Figure 2:** Antiviral activity: concentration- inhibition response curve of the inhibition of cytopathic effect of four fold dilutions of measles virus by rosemary extract

Interestingly, sage showed a dose dependent inhibition of Measles virus cytopathic effect at all virus dilutions as shown in Table 2 and Figure 3. A good correlation at the second dilution of virus ( $r^2=0.976$ ) and  $EC_{50} = 14.74$  µg/ml was achieved as illustrated in Figure 4.



**Figure 3:** Antiviral activity: concentration-response curve of the inhibition of cytopathic effect of four fold-dilutions of measles virus by sage extract



**Figure 4:** Antiviral activity: concentration-response curve of the inhibition of cytopathic effect of four fold-dilutions of measles virus by sage extract

#### 4. Discussion

The present study aimed to investigate the inhibitory effect of two different extracts of commonly used medicinal plants in Jordan against Measles virus using Vero cell line.

Rosemary and sage extract showed a promising antiviral activity against Measles virus with a dose-dependent inhibition of the different concentrations of sage on a measles virus cytopathic effect at all virus dilutions. The

findings of this work can be explained depending on the previous studies that reported the antiviral activities of the different constituents present on the plants which were tested in this work; so that the antiviral activity of the two plants might be related to the presence of polyphenols compounds such as carnosic acid and rosmarinic acid which are shared between them [4].

Reference [23] was the first concluded that polyphenols act principally by binding to the virus and/or the protein of the host cell membrane and thus arrest absorption of the virus. Moreover in a study of screening potential antiviral agents from wide range of natural products. Reference [24] figured out that viral inactivation in vitro is directly attributable to preferential binding of the polyphenols to the protein coat of the virus. Furthermore, polyphenols antiviral activity may be clarified by their ability of clumping the virus Particles together into complexes, which are largely non infective [25]. Therefore enveloped viruses may be the most vulnerable to the action of polyphenols easily by interacting with the glycoproteins of the viral envelope.

Carnosol is another natural occurring polyphenols found in rosemary exhibited definite anti-HIV activity at an early stage of virus infection, since HIV is an enveloped RNA virus like MV; carnosol could be responsible for rosemary antiviral activity [26].

Rosemary contains other phenolic compounds such as caffeic acid, eugenin and apigenin which may act by different mechanism that ended with inhibiting viral RNA replication. These phenolic compounds might be one of the bioactive components that are responsible for the antiviral activity alone or in a synergism with other compounds; In addition, luteolin found in rosemary exhibited high and concentration-dependent levels of antiviral activity against HSV-1 strains (enveloped RNA viruses like measles virus) prior to and during adsorption stage [27]. Accordingly antiviral activity of rosemary extract may be related to the presence of large quantities of polyphenols compounds. A finding that offered new insight regarding to the biological activity of ursolic acid which belongs to triterpenes and presents in rosemary, suggest that ursolic acid suppressed the growth of human papillomavirus (HPV)-associated cervical cancer cells [28]. 1, 8-cineole,  $\alpha$ -pinene the main constituents of rosemary essential oils revealed antiviral activity through inhibition of viral replication [29].

In addition to the presence of polyphenols that may possess antiviral activity against MV, sage antiviral activity may be due to the presence of flavonoids and terpenes that showed antiviral activity [30]. Another finding pointed out the presence of two new diterpenoids, safficolide and sageone in sage, also showed antiviral activity [24].

Sage essential oils contain 1, 8-cineole,  $\alpha$ -pinene too which revealed antiviral activity through inhibition of viral replication [29]. In view of that it is seemed that the two plants possess antiviral activities which mainly may be due to the presence of flavonoids, polyphenols and terpenes.

## **5. Conclusion**

This study provides evidence that rosemary, sage plants which are used by the Jordanians to maintain health could have potential antiviral activity against measles virus. It is possible that the elucidation of active constituents in these plants may provide useful lead to the development of new and effective antiviral agents.

However, the results show for the first time the antiviral activity of these plants against *measles* virus. Also the study suggests that there might be a shared active compound or compounds with closely related chemical structures between rosemary, sage that inhibit *measles* activity.

It is recommended that testing of each constituent present in the extracts will be necessary to identify the active substances since it is not clear which plant constituents, singly or in synergy, are responsible for the observed anti measles activity, also the elucidation of the mechanisms underlying antiviral effects on *measles* virus was beyond the scope of this study, to investigate the effect of the plants on different steps of viral replication the preparation should be added at varying times relative to viral infection( the timing of addition of plant extracts to the cells before with or after adding the virus titer can investigate the possible mode of action of them , effect on adsorption or effect on penetration) .

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