

## Assessment of Antiviral Activity for *Lamiaceae* Family Members Against Rna and Dna Virus Models Using Cell -Culture: in Vitro Study

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**Abstract:** Plants belonging to family *Lamiaceae* are traditionally used in many aspects in the Middle East and worldwide which gave them a great value for studying its usage during management of infectious diseases. Aqueous extracts of dried leaves from well known species of family *Lamiaceae* were examined for their inhibitory potential against RNA virus models namely Measles, Mumps, Vesicular Stomatitis Virus (VSV) and DNA model; Herpes simplex type 2. Data recorded revealed that sage (*Salvia Officinalis*) at concentration of 1500 µg/ml, Rosemary (*Rosmarinus officinalis*) at concentration of 500 µg/ml and Thyme (*Thymus vulgaris*) at concentration of 2250 µg/ml showed antiviral potentials. Data recorded revealed Antiviral potentials of test extracts against RNA can be arranged in the order of sage followed by rosemary and thyme. On other hand on using VSV as a animal RNA virus model, rosemary showed the highest antiviral potential followed by thyme and lastly sage. While antiviral potential against DNA virus model; HSV- II was detected and arranged in the order of rosemary followed by thyme while sage showed no antiviral potential. The obtained results indicate a promising inhibitory activity of plant extracts against various virus models except for sage which showed no inhibitory activity against HSV-2 even though it showed reactivity against other virus models. Chemical analysis of extracted bioactive materials showed that the elementary analysis of structure of rosemary was C,79.3;H,13.3;Cl,5.09;O,2.3, thyme: C,66.10;H,10.77;I,20.54;O,2.59 and Sage: C,67.93; H, 10.80;I, 18.89;O,2.38.

**Key words:** Sage • Rosemary • Thyme • Antiviral • Measles • Mumps • VSV • HSV

### INTRODUCTION

Antiviral drugs may not be a perfect choice in many cases due to their toxicity as they may affect both the virus as well as the host cell besides the fact of virus resistance development to the drug due to mutation and conflicting efficacy in recurrent infection in immune-compromised patients [1]. Although vaccination is also a choice for the control of some viral infections, some viral infections are still widely spread especially among children though vaccination. For instance, in United Kingdom mumps vaccination programme had

experiences of large scale outbreaks [2, 3]. Similarly, in Egypt during October 2012 although the children have received at least one dose 3500 children were suffering from mumps. This tragedy is the same in measles which is the leading cause of death among young children where, although effective vaccine is developed but according to WHO 15% fail to develop immunity against measles prior to vaccination [4]. Therefore, eradication of mumps and measles may be more difficult and these two serious diseases may represent a real threat for another era. This urged the need for new approaches to manage viral infectious diseases using medicinal plants or other

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compounds of natural origin. In this regard, WHO has defined medicinal plants as the plants that contain properties or compounds that can be used for therapeutic purposes [5]. Studies conducted in laboratories around the world revealed that medicinal plants can provide a rich source of antiviral activities [6-13].

In this prospective, the current study aimed to screen the antiviral activities of some plant extracts against those two RNA viruses using vero cell model and evaluation for the antiviral activity done using MTT assay and also by measuring the loss in virus which was determined by the difference between the virus titers of control and treated viruses ( $\bar{a} \log_{10} \text{TCID}_{50}/\text{ml}$ ) [14]. Reed and Meunch [15] calculation method used for determining the virus titer by cell culture titration method. The plant extracts in this study that are used for treatment of Vero cells prior to infection with the virus are aqueous extracts of three plants belonging to family: Lamiaceae namely sage, rosemary and thyme. Sage traditional use in mouth and throat inflammation has been justified in a number of studies [16-19] as well as its antimutagenic and cancer preventive activities been reported [20 - 22]. It also been reported for its antiviral activity against HIV [23] and VSV [24].

Rosemary is also known for its anti leukemic effect [25] and its antiviral activity against HIV [26] as well as its antiviral activity against HSV-I in a clear concentration dependent manner. Thyme similarly which is well known herb in the Middle East countries is also reported for its anthelmintic (especially hookworms), antibacterial and antifungal properties [27] and antiviral activity against HSV [28].

## MATERIALS AND METHODS

**Preparation of the Sterile Aqueous Extract:** 10 g of the dried leaves were added to 100 ml previously boiled distilled water and left for 15 minutes at room temperature to cool then filtered using Whatman® filter paper. The filtrate was then sterilized using 0.2µm syringe filter (Millique, USA). Sterile aqueous extracts were aliquoted in 2 ml Grenier® cryo tubes and preserved at -40°C (Sanyo biomedical freezer, Japan)

**Preparation of Vero Cells:** Vero cells (African green monkey kidney cells) clone CCL-81 from ATCC was kindly supplied from cell culture dept. the Egyptian holding company for vaccines and Sera (VACSERA) as a confluent sheet of healthy bright cells prepared using medium 199-E according to the protocol of manufacturing.

Medium was supplied from Sigma-Aldrich, USA) Vero culture was prepared in 96-well tissue culture plates (Corning®). 24 h post incubation at 37°C under a humidified 5% CO<sub>2</sub> atmosphere (Thermo scientific® CO<sub>2</sub> incubator, Germany)

**Cytotoxicity:** Cytotoxicity evaluation was performed using MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [29, 30]. Precultured T.C plates growth medium was decanted and test materials were dispensed to the plate wells in a descending manner as 0.1 ml / well. Test material were diluted in a 2% FCS and 1% units /ml Penicillin 1% µg/ml streptomycin (10000 units /ml Penicillin 10,000µg/ml streptomycin) maintenance medium. 48 h post cell treatment, treatment medium was decanted from TC plates and 50 µL of 1% MTT were added and plates were incubated once more for 4 h. MTT containing solution was removed without disturbing the cells, 50 µl of DMSO (Prolabo®) were added to each well to dissolve the Formozan crystals. After gently shaking the plates for 15 minutes using plate shaker (Stuart®- UK), the crystals were completely dissolved and the absorbance were read on a multiwell spectrophotometer (BioTek®, USA) at 570 nm and the average OD for the remaining cells for each extract dilution was measured. Viability% was determined according to the following equation:  $\text{Viab.}\% = \frac{\text{X OD of test sample}}{\text{X OD of cell control}} \times 100$

Viability % was plotted against test extract concentration / dilution IC<sub>50</sub> values were presented as the percentage of survived cells compared to control cells and the highest non- toxic dilution was determined [31]

**Evaluation of the Antiviral Activity of the Plant Extracts Using Mtt Assay:** Vero ( $2 \times 10^5$  cells/mL) were prepared in the same way as described above. Growth medium decanted and 100 µl/well of non-cytotoxic concentrations of the extracts as well as interferon (Pegintron®) added and incubated for 24 hours. Various dilutions of virus models were dispensed as 100 µL/well [32]. Negative cell control and viral controls were included. Plates were incubated till the cell show cytopathic effect (CPE) and MTT method used as previous [29] and [30]. The percentages of protected cells was calculated in comparison to that achieved under the effect of interferon

**Evaluation of the Antiviral Activity of the Plant Extracts Using End Point Assay:** Vero cell cultures ( $2 \times 10^5$  cells/mL) were prepared as previous according to [33] the highest non- toxic dilution of the extract was used for 24h cell treatment. Treatment medium was

decanted and 10-fold dilutions of the virus were added to the respective wells. Measles, Mumps, VSV and HSV2. Infected Pre treated plates were microscopically examined using inverted microscope (Olympus®, Japan) for detection of cellular changes / cytopathic effect (CPE) and the scored CPE was recorded and the virus titer was calculated using Reed and Meunch (1938) method [15]. The antiviral activity was measured as the difference between test materials treated cell virus titer and non-treated cell virus titer. The depletion rate was expressed as the percentage loss in the virus titer. The experiment was done in triplicate series in order to measure the difference using ANOVA.

**Chemical Analysis for Extracted Compounds:**

Elemental analysis of the three aqueous extracts by UV (using The CAMMAG TLC scanner system) IR (using anicum infinity series FTIR, Perkin-Elmer 1650 spectrophotometer)-HNMR spectroscopy (using Proton Nuclear Magnetic Resonance spectrum EM-5000 MHZ spectrometer using (CHCl<sub>3</sub>) as organic solvent) and mass spectroscopy (using Direct Inlet part DI-50 to mass analyzer in Shimadzu GC/MS-QP5050)

The analysis was done for predication of the chemical structure at micro analytical center faculty of Science Cairo University, Egypt.

**Statistical Analysis:** The means of loss in the virus titer, between treated and non-treated groups were compared using one way ANOVA descriptive tests and P value was set < 0.05 for all analyses.

**RESULTS**

As the aim of the work is mainly to evaluate the inhibitory effect of plant extract to test viral models namely Mumps and Measles, the virus loss reflects the resistance of the cells to the virus infection and this resistance is attributed for being previously treated. The aqueous extracts of Sage, Rosemary and thyme were generally safe to Vero cells at any dilution (Fig. 1) using MTT assay at which OD and the % of cell viability showed that cell viability resembled that of nontreated cell control and no morphological changes recorded. Upon comparing the antiviral activity of the three aqueous extracts against Mumps and Measles to the activity of interferon are almost the same for Rosemary at 1000,500,250 and 125µg/ml; Sage at 3000, 1500, 750 and 375 µg/ml and Thyme at 4500, 2250, 1125 and 560µg/ml. Higher dilutions showed limited activities comparing to interferon [Fig 2-3] for Mumps and Measles respectively. So, furthermore comparative evaluation was processed to measure the average loss of the virus titer as percentage loss (% depletion rate) which reflecting the antiviral potential of plant extracts on Vero treated cells compared to only virus treated cells. Data recorded showed that the three extracts results in noticeable loss in virus infectivity titer in case of pre- treatment of the Vero cell with the extract prior to infection with Mumps or Measles virus and the loss is almost the same upon treatment of any of the three extracts, that mean the extracts may have a certain mismatching effect on the cell membrane configurations leading to suppressing viral entry or may

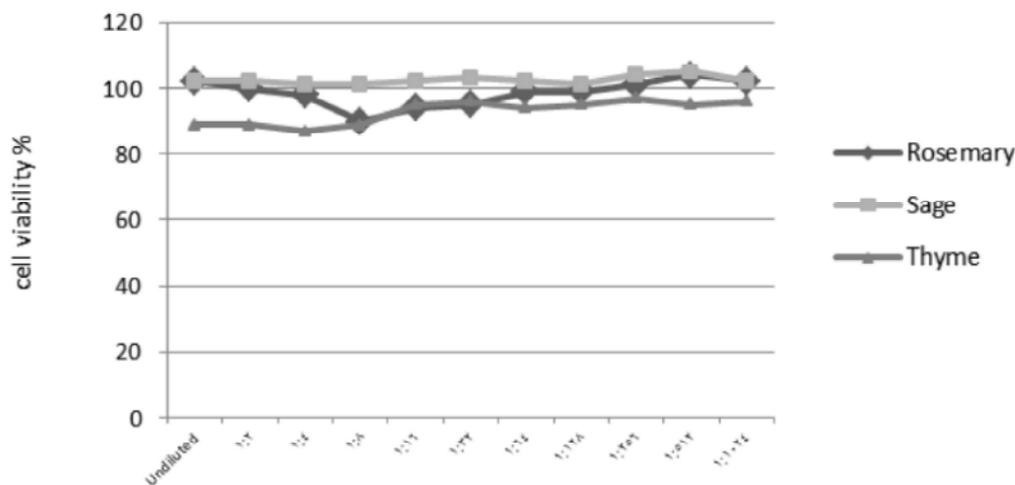


Fig. 1: Evaluation of viability % of plant extracts using MTT assay

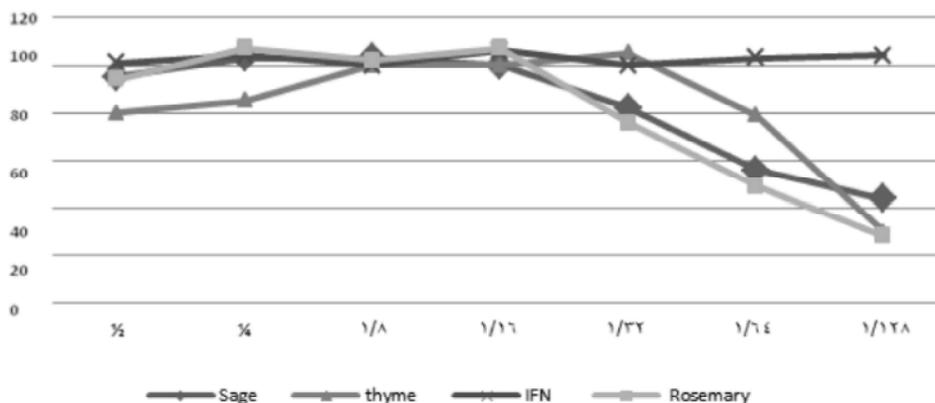


Fig. 2: Evaluation of antiviral activities against Mumps Virus post treatment with the extract using MTT assay relative to Interferon

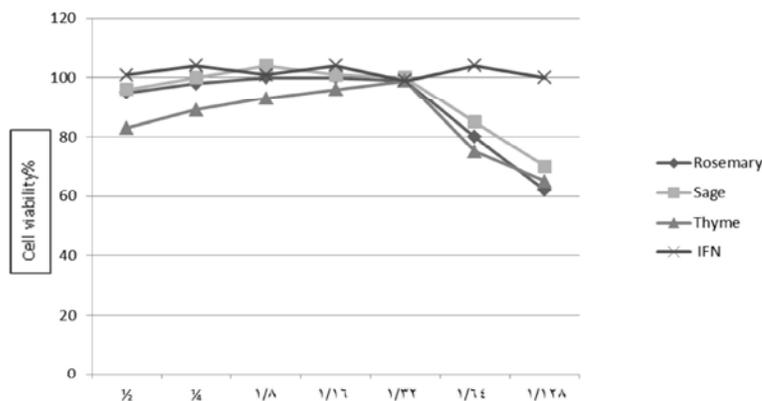


Fig. 3: Evaluation of antiviral activities against Measles Virus post treatment with the extract using MTT assay relative to Interferon

Table 1: Showing the depletion rate of each virus that resulted from pre-treatment of Vero cells by the plant extract

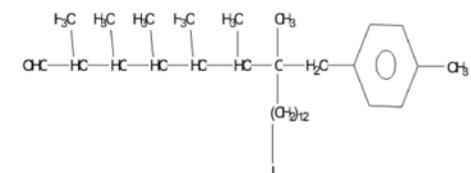
Aqueous extract/concentration	X virus titer without treatment log <sub>10</sub> TCID <sub>50</sub> /ml				X virus titer with treatment log <sub>10</sub> TCID <sub>50</sub> /ml				X(Δlog <sub>10</sub> TCID <sub>50</sub> /ml)				Depletion rate %			
	Mumps	Measles	VSV	HSV-2	Mumps	Measles	VSV	HSV-2	Mumps	Measles	VSV	HSV-2	Mumps	Measles	VSV	HSV-2
Rosemary 500 µg/ml	5.55	4.44	6.22	7.26	3.9	3.06	3.05	4.64	1.61	1.38	3.17	2.62	29	31	51	36
Sage 1500 µg/ml					3.71	2.77	3.99	7.2	1.88	1.67	2.23	0.06	33	37.7	35.9	0.8
Thyme 2250 µg/ml					4.1	3.1	3.64	5.56	1.43	1.32	2.58	1.7	26	29.8	41.5	23

have an effect on one of the enzyme responsible for viral cell entry. Other two animal / human virus models (VSV as RNA virus model and HSV-2 as DNA virus model) were used for evaluation of antiviral activities based on 24 hr pretreatment of Vero cells with test materials, the three extract show noticeable loss in the virus infectivity titer except for Sage showed no effect on infectivity of HSV-2 (Table 1). So, Sage at concentration 1500 µg/ml showed the best inhibitory activities against Mumps and Measles (virus depletion rate was 33 % and 37% respectively) followed by Rosemary at concentration 500

µg/ml resulted in virus depletion of Measles and Mumps (31 % and 29% respectively) and Thyme at concentration 2250 µg/ml has showed a slight less inhibitory activity than Sage and Rosemary against Mumps and Measles (virus depletion 26 % and 29.8% respectively). On other hand Rosemary showed the highest inhibitory activity against VSV as the virus loss was more than 50 % indicating that Rosemary has a great inhibitory activity against VSV followed by Thyme recording 41.5% loss in VSV virus titer while Sage has shown the least inhibitory activity against VSV (35.9%) but still its inhibitory activity

is pronounced but less than Rosemary and Thyme. HSV type 2 was apparently more resistant to the plant extracts used in this study except for Rosemary at concentration of 500 µg/ml resulted in a loss of 36% and to lesser extent Thyme at concentration of 2250 µg/ml showed a depletion rate of 23 % and it is clear that the resulting depletion is much less than other viruses on other hand HSV type 2 is quite resistant to Sage as there was almost no virus loss. Elementary analysis of test materials was represented (Fig5-7) revealed that the antiviral potential may be attributed to the different chemical groups attached to toluene ring or NO of methyl groups in branched carbon chain

**Thyme:**



2,3,4,5,6,7-hexamethyl-7-(4-methylcyclohexylmethyl)-19 λ<sup>3</sup>-nonadecanal compound with iodine (1:1)  
 Chemical Formula: C<sub>33</sub>H<sub>56</sub>Cl  
 Exact Mass: 617.42  
 Molecular Weight: 617.81  
 m/z: 617.42 (100.0%), 618.42 (37.0%), 619.42 (6.8%)  
 Elemental Analysis: C, 66.10; H, 10.77; I, 20.54; O, 2.59

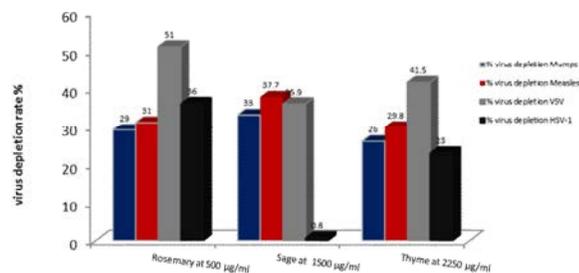
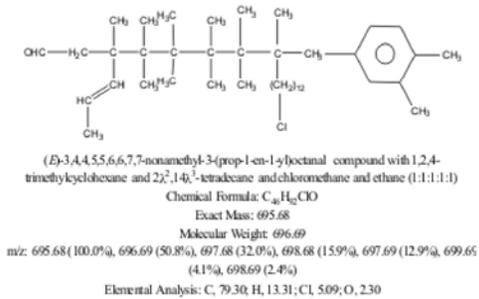


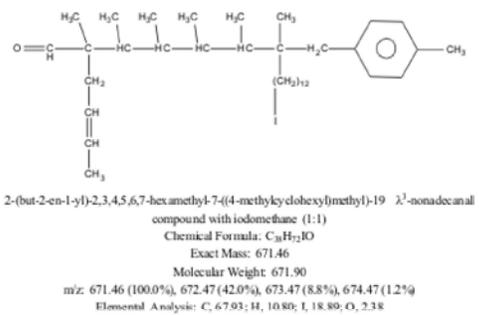
Fig. 4: Comparative evaluation of the three plant extracts

Predicted formula for the expected active extract: [Fig :5- 6 7]

**Rosemary :**



**Sage:**



**DISCUSSION**

Data recorded in the present study regarding antiviral potential of aqueous extract was in consistent with a study [34] recording Rosemary, Sage and thyme belonging to family Lamiaceae which is also known as mint family and this family is reported in many studies that it produces variety of active constituents with medicinal properties and that several members to this family have been reported to have antiviral activity including lemon balm (*Melissa officinalis* L.), sage (*Salvia* spp.), peppermint (*Mentha × piperita* L.), hyssop (*Hyssopus officinalis* L.), basil (*Ocimum* spp.) and self heal (*Prunella vulgaris* L). Also, the variability of antiviral potential may be attributed to the part of plant used in extraction process and type of solvent polarity.

In the mean times our data was in agreement with [35-37] concerning the viral model HSV recording that the aqueous extracts of *Prunella Vulgaris* contain more antiviral activity than did ethanol extracts, displaying potent antiviral against HIV-1 indicating that polar constituents are important for the antiviral activity. These findings are consistent with previous antiviral observations made with *P. vulgaris* extracts in studies against EIAV and HSV

Also the aqueous extracts from peppermint, sage and lemon balm leaves were examined against HIV [23] and those aqueous extracts from Lamiaceae show drastically and rapidly reduce the infectivity of HIV-1 virions at non-cytotoxic concentrations. An extract-induced enhancement of the virion's density prior to its surface engagement appears to be the most likely mode of action. By harboring also a strong activity against herpes simplex virus type 2, these extracts may provide a basis for the development of novel virucidal topical microbicides.

Similarly a study on the antiviral activities of the aqueous extracts [28] from lemon balm (*Melissa officinalis*), peppermint (*Mentha x piperita*), prunella (*Prunella vulgaris*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*) and thyme (*Thymus vulgaris*) against HSV-1 and HSV-2 and acyclovir resistant strain of HSV-1 and all the extracts have shown the extracts exert their antiviral effect on free HSV and offer a chance to use them for topical therapeutic application against recurrent HERPES infections and agreed with the fact that the aqueous extracts of family Lamiaceae are of inhibitory effects against viral infections when assessed in in-vitro studies. So, in this study the aqueous extracts were used as their antimicrobial activities may attribute to their constituents. Also the use of solvent as water makes the results more reliable for any possible application of the plants under study. On other hand and regarding the type of solvent and related extraction efficacy, many studies have conducted the antiviral assays using methanolic or ethanolic extracts rather than aqueous extracts as [38] in which the extracts of Sage (*Salvia officinalis*) were obtained by fractionating ethanol extracts at different pressures and the extracts under evaluation using wish-VSV model have shown viral reduction. The convenience of the use of aqueous extract in this study besides being it the first study for the antiviral activities of plants belonging to family Lamiaceae against Mumps and Measles are the main reasons for the choice of aqueous extract rather than methanolic or ethanolic extract. Similar studies are done [32,40] for the antiviral activities against measles only using Malaysian plants and Nigerian plants respectively and in each study the plants under studies show antiviral activity against measles virus. Another study [41] evaluated the effect of *Melastoma malabathricum* extracts which is widely used in Malaysia. And has proved that Cells treated with simultaneous addition of Measles virus and MMME at 0.1 and 1 LC50 were found to survive from viral infection. The antiviral effect of the that plant extract is probably due to the quercetin content that can inhibit reverse transcriptase [42] which is the early part of the measles' replication process.

The first thing was done before the screening of the extracts was to evaluate the cytotoxicity of the extracts on Vero cells [43] as if the extracts are too toxic, the antiviral results are not valid because it will have a low therapeutic value (treatment value: toxicity level ratio) and as shown in fig (1) the extract was generally safe at any

concentration but thyme shows less safety on Vero cells according to MTT assay if compared to Sage and Rosemary but still described as generally safe. Most of the studies have carried out the cytotoxicity evaluation have used MTT assay [29-31] with little modifications and also cytotoxicity evaluation could be done by observation of morphological changes in situ [44] in both methods the cytotoxicity evaluation was done to evaluate the cytotoxicity of the extracts to the cell line used in the study only in this case Vero cell line.

Another study [32] has extended the evaluation of the toxicity of the plant extract using Brine shrimp lethality bioassay in which the toxicity of the different extracts was tested by lethality to *Artemia* saline brine shrimp [45]. Concentrations of 10, 100 and 1000 ppm of each active extract were tested. The number of dead larvae was recorded and used to calculate the 50% lethal concentration. This technique is rarely used as it is hard to apply in most of the conducted studies and the use of the cell line is more common.

Evaluation of the antiviral activities differ from author to another in this study we used both MTT assay and virus titer log reduction method by calculating the difference between virus titer of the control virus and its titer when it is used to infect extracts pre-treated cells with the extract. MTT method is used for evaluation of South American plant extracts against herpes simplex virus [46] and for evaluation of two Indian plants against Mumps and measles [47] and the principal was almost the same as that used except that a standard antiviral agent is used in the current study which is the interferon. The antiviral effect evaluation was done using mainly Mumps and Measles by the MTT assay and the three extracts has shown remarkable antiviral activities when compared to interferon and further evaluation was done by evaluation of the virus loss using tissue culture titration technique In both techniques 24 hrs pre - treatment of the cells by the extract prior to the infection which shows if the plant is able to protect the cells against infection [48] the prophylactic action involves interferon activity from the cells which can be induced by viral infection or metabolite in the extract. Several authors had conducted studies to evaluate plant extracts against measles or mumps. In the present study evaluation done against both viruses due to their closeness in nature as they are both single stranded an enveloped RNA virus in the family Paramyxoviridae also due to their importance as described before.

Activities of many extracts of plants that are endogenous to several countries were evaluated against Measles and Mumps using different methods [47] and the study have shown that the active principle component from Terminalia chebula fruit and Semicarpus anacardium nuts (plants endogenous to India) have no cytotoxic activity against VERO cell line and also showed relatively high antiviral activity. Both the medicinal plants found to be a good antioxidant agent when compared to standard synthetic antioxidant Butylated Hydroxy Toluene (BHT). The plants used for this study was found to contain phenols as a photochemical which was demonstrated by Gas Chromatography-Mass Spectrometry (GC-MS). So there may be a correlation in phenols being responsible for all the activities.

### CONCLUSIONS AND RECOMMENDATIONS

The above results suggest that the aqueous extracts of Rosemary, Sage and thyme could enhanced the reduction of Measles and Mumps infectivity and further investigation are recommended

Rosemary and Thyme is also to be considered in genital diseases as HSV type 2 when used in a generic formulation

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