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Anti-respiratory syncytial virus activity of clove extract

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Abbreviations

NP   Natural products

RSV   Respiratory syncytial virus

Abstract

Background

Natural products have been used by humans for thousands of years for healing purposes. They occasionally resulted in the development of research-proven new drugs. A well-known example of such a drug is the malaria medicine artemisinin isolated from the plant sweet wormwood. Respiratory syncytial virus (RSV) is an RNA virus that belongs to *Paramyxoviridae* family and is known for causing severe respiratory infections in new-borns and elderly. As of today, there is still no registered drug available against this important virus nor a vaccine for its prevention. With lack of effective antiviral therapy, treatment of acute RSV mainly involves supportive care.

Aim

To investigate whether any specimen of a collection of traditional medical plants exhibits antiviral activity against RSV in cell culture.

Methods

A collection of traditional medical plant was pulverized and extracted with sterile water to be screened for antiviral effect against RSV. The hit plant, a clove flower bud, was studied in more details to determine its anti-RSV potency, toxicity for HEp-2 cells, and the mechanism of antiviral activity.

Results

Out of several plants examined only clove extract showed anti-RSV activity. This activity was manifested by inhibiting RSV infection of HEp-2 cells with IC50 value of 102 µg/ml and reducing, at 2000 µg/ml, the viral yield by ~10000 times. The activity was highly selective for RSV and directly targeted the free RSV particles infectivity during early step of viral infections.

Conclusions

Clove extract showed a potent anti-RSV activity that appeared to be virucidal. Further extension of these studies based on identification of active ingredients of clove extract is warranted for its development as anti-RSV drug or as an air disinfectant.

Introduction

Natural products and drug discovery

Natural products (NP) are substances or compounds that are found in nature or are produced by living organism such as bacteria, plants and animals. These products contain bioactive molecules that frequently exhibit complex structures [1].

NP have been used by humans for thousands of years for healings purposes and based on archaeological studies, the application of medical plants dated back to 2600 BC [2]. Ancient civilizations and theirs so-call shamans or traditional healers experimented with various type of plants through trails and error on humans to determine the effect they might have against diseases. Plants with notable therapeutic effect are then passed down to next generations [3]. The medical practice of the ancient Egypt and its sister civilisation Nubia of northern Sudan is some of the oldest documented. Egyptian medicine was highly advanced for its time, including surgical and dental procedure and an extensive drug-making. The ancient Egyptians discovered many medical plants and made recipes to help heal different health conditions. It is mentioned for instance in the Egyptian medical papyri that honey with cloves was used as a remedy against cough and upper respiratory symptoms. Later, the Egyptian influenced the Greeks and other traditions and such recipes with medical plants like cloves further developed and found more use[4, 5]. In Europe, pomander, a famous aromatic Christmas decoration and gift that is made of orange with stubbed cloves, was used in 17th century as protection against airborne infections and bad odor [6].

As of today, clove like many other medical plants are still broadly used. It is estimated by the world health organization (WHO) that 80% of the population of some countries in Asia and

Africa are using herbal medicines for some health care aspect. Traditionally the practise of herbal medicine and NP was linked to traditional healers that passes the recipes though generations, but today, herbal remedies are available and distributed in many health stores throughout the world with many forms of preparations and ways of administration [7].

Despite the popular use of NP and medical plants, there are many challenges associated with them regarding reliability, safety, lack of support by scientific evidence, and their potential misleading or anecdotal health advice. Medical plants are often likely to cause adverse effects or interaction with approved drugs, and moreover many plants can be very toxic thus requiring regulation by health authorities [8]. In the EU, herbal medicines are regulated under the committee on herbal medical products [9]. In Sweden, herbal medicines or any NP for medical uses are regulated by Swedish medical products agency (läkemedelsverket) [10].

However, NP especially medical plants are well-documented sources of biologically active molecules, and their contribution to the field of pharmacology reaching approximately 25% of all modern drugs [8]. Classical examples of NP-based drugs are analgesics like salicylic acid originally from the bark of the willow tree and opium from poppy plants. Anti-infectives with penicillin have been the first antibiotic and the most revolutionary that saved millions of people critically affected by bacterial infections [11]. Several inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG_CoA) reductase lowering cholesterol level in the blood, nowadays known as statins were originally derived from NP. Furthermore, hypertensions drugs like captopril, an angiotensin-converting enzyme inhibitor, was isolated from Brazilian arrowhead viper plants [12] [11]. Regarding the field of virology, silibinin, a compound from the milk thistle plant has been approved for treatment of hepatitis C virus [13].

Despite these contributions that NP provided for drug discovery, numerous challenges limited

their use in research, including the decline in interest from the pharmaceutical companies in the 21 centuries. This is believed to be caused by the use of the high-throughput screening of synthetics of compounds which resulted in rapid discovery of new hit compounds followed by the design, synthesis and evaluation of optimized candidate drugs, i.e., features superior to the need of tedious isolation and identification of active compounds from NP [11].

Respiratory syncytial virus, a virion structure and a viral disease

Human respiratory syncytial virus (RSV) is an RNA virus that is known as a cause of acute infection of lower respiratory tract [14].

Viruses are infectious particles which have ability to cause disease in living organisms. They come in different structures depending on the type and the species but in general all viruses consist of nucleic acid (DNA or RNA) protected by covering protein frequently in form of icosahedral box known as capsid, and furthermost lipid envelope with embedded viral spikes. Viruses initiate infection of host by attaching to susceptible cells followed by entry into the cells though cell plasma membrane resulting in release of their genome and use of host cell nucleotide and protein synthesis machinery for their reproductions. This process causes cytopathic effects in the cells in the form of destruction, plague formation or other cellular abnormalities [15].

Symptoms of viral infection appear either through direct cell destruction and/or inflammation, i.e., when the immune system start to target the infected cells resulting in cell and tissue damage. Some viruses cause acute infection with self-clearing illness, other like HIV cause chronic infections. Viruses may also get seemingly inactive after causing acute infection for a period of time and then reactive again when the host immunity is no longer activated. This phenomenon is called viral latency and typical example of it is herpes simplex virus [15].

Viruses can cause disease in almost every organ in the body but more frequently in the respiratory system because respiratory viruses are highly contagious due to fast and efficient airborne spreading [16].

Humans are frequently affected by common colds viruses, these viruses are characterized by similar set of mild upper respiratory tract symptoms like nasal obstruction, sneezing, coughing and sore throat. In humans, common colds are usually caused by rhinovirus [17]. Other respiratory viruses like RSV, influenza, parainfluenza, and coronaviruses can also to varying degree cause cold symptoms, but these viruses often associated with more severe symptoms and sometimes with fatal outcomes because of their ability to spread into the lower respiratory track [18].

The severity of respiratory viruses' infections depends on various factors like, tropism for respiratory cells, barriers of the respiratory tract and efficiency of the host immunity. This means that, induvial with underlying lung disease, comorbidities, premature birth, immunosuppression, immature immune response (new-borns), and inefficient immunity (elderly) are at a higher risk for serve respiratory infection and complications [19].

RSV is a middle sized and enveloped virus particle that contains negative-sense, single-stranded RNA. RSV belongs to *Paramyxovirade* family. Its name reflecting the virus-induced cytopathic effect in cultured cells, i.e., merging of numerous infected cells into so-called syncytia due to capability of its surface F-protein to cause fusion of cell plasma membranes of neighbouring cells. RSV consist of 10 gens that code for 11 proteins, among these is G protein and the F protein which are involved in the attachment of the virus to and entry into susceptible cells. These proteins are embedded in the lipid envelope, and their endings below the lipid envelope contact the matrix (M) protein. The M protein is the scaffold component of

the virion that also connect to the viral nucleocapsid, i.e., the viral N protein located along the surface of viral RNA [20].

This virus can cause severe bronchitis followed by airway obstruction in children < 5 years, immunocompromised individuals and elderly. In healthy adult's RSV cause mild cold symptoms or appears non-symptomatic [21]. It spreads through air by droplets and by direct contact with infected fluids [22].

RSV is wildly spread across the globe and has its pick during the winter months in Europe and rainy months in tropical countries. In the year 2015, it caused about 66,000-199,000 death among children <5 years in low-income countries [23]. In Sweden, the public health authority (folkhälsomyndigheten) noticed an increasing trend in the number of RSV diagnosis with 700 cases been reported this year [24].

As of today, there is neither a specific and effective antiviral therapy against RSV infection nor vaccine for its preventions, and therefore the treatment of acute RSV mainly involves supportive care, such as administration of intravenous fluids, oxygen therapy and mechanical ventilation. Immune prophylaxis with RSV neutralizing anti-F monoclonal antibodies can be given to children at high risk groups but these antibodies are very expensive and have very restricted indications.

Unlike many of the childhood's viruses, RSV lack an effective vaccine, despite many years of clinical and preclinical studies. One of the vaccine candidates in 1960 resulted into so-called vaccine enhanced diseases with a deadly outcome. Children that received the vaccine exhibited more severe illness upon natural reinfections than those in the control groups. [14]

Several attempts have been done to find a drug against RSV. These include screening of small-molecule for targeting some of the important components of the RSV particle,

especially the G and the F glycoproteins but none of these molecules have resulted in an approved drug as yet, mainly due to insufficient activity and/or safety issues [25] [26].

Today the only approved antiviral against RSV is the nucleoside analog ribavirin. This drug is administered as aerosol mainly in the hospital settings. Its mechanism of actions is not fully understood but it was reported to have a broad inhibitory spectrum against a range of RNA and DNA viruses. Although ribavirin is the only registered drug against RSV its efficiency is ambiguous and a matter of debate [27].

Given the severity of RSV disease in children and elderly, and its greatly contagious character with high prevalence in human population, there is a need for development of anti-RSV drugs. In the investigation presented in this thesis screening of traditional medicine plants for anti-RSV activity was performed and resulted in identification of specific virucidal activity of clove extract against RSV infectious particles.

AIM

The aim of this preclinical study is to investigate whether any specimen of a collection of

medical plants from the current Nubian traditional medicine have antiviral effect against RSV in cell culture. If so, we want to determine the mechanism of actions of the antiviral activity of the hit plant at a basic level, i.e., whether the activity is virucidal or virustatic, whether it concerns cellular or viral components essential for RSV replication, and where in the virus cycle this activity takes place. Also, we will study the cell toxicity of the hit plants showing antiviral effect. Our goal is to help find a new antiviral candidate against RSV by identifying an effective natural compound that can be a subject for future studies and isolation of its effective molecule.

Material and methods

Ethics. No ethical permits are needed for this study since no humans or animals were involved.

Plants. The following plants material was used. Fenugreek (seeds), Green Cardamom (seeds), Cloves (flower buds), Baobab (fruit and seeds), Tamarind (fruit and seeds), Roselle (flower petals) and Acacia (seeds in pods). These plants were recommended and bought from Omdurman city market in Khartoum, Sudan, based on presumed use among the locals in treatment of upper respiratory infections.

Media. Unless otherwise stated, the following media and supplements were used. Dulbecco modified Eagles medium (DMEM). Fetal bovine serum (FBS) that was heat inactivated by

incubation for 30 min at 56° C (HI-FBS). Stock of penicillin and streptomycin (PEST) comprising 10 mg/ml of streptomycin in Hanks medium. Stock of L-gulatamine containing 29.2 mlg/ml glutamin in deionized water. HEPES stock (0.4 M) in Eagles medium. Methyl cellulose stock containing 1.5 g of methyl cellulose in 100 ml of Hanks medium, that had been sterilized by autoclaving at 120° C for 20 min. One% crystal violet stock solution was prepared by dissolving 1g of crystal violet in 87 ml of 70% ethanol supplemented with 8.7 ml of formalin and 4.3 ml of acetic acid.

Viruses and cells. Human laryngeal epidermoid carcinoma (HEp-2) cells (ATCC, CCL-23) were propagated in Eagles medium supplemented with 8% FCS. RSV strain A2 [28] (ATCC, VR–1540) was propagated in HEp-2 cells [29], and the virus stock comprising 25% sucrose [30] was stored at -80° C.

Preparation of plants extracts. The plants material was pulverized in a mortar and then extracted with a sterile redistilled water by vertexing and incubation for 1 hour at room temperature. The suspension was then vortexed again and centrifuged at 5000 x g for 10 min at room temperature. The resulting supernatant was aliquoted and stored at -20° C.

Determination of anti-RSV activity of plant extract. Two separate methods relied on determination of inhibitory concentration of plant extract that reduced the number of viral plaques by 50% (IC50) and the viral yield reduction assay were used. The viral plaque reduction (IC50) assay was performed as follows (Lundin et al., (2013). Briefly, the day before the experiment, HEp-2 cells (1.2×10^5 cells/ml) were seeded in 12 well plates to became approximately 70% confluent. Next, serial 5 folds dilutions of plant extract (ranged from 16 to 50000 µg/ml) in DMEM medium supplemented with 2% HI-FCS, 1% L-

glutamine, 1% PEST, and 1% HEPES stocks (DMEM-M) were performed in an empty 24 well plates, then mixed with 100 μl of RSV-A2 dilution comprising 300 plaque forming units (PFU) and incubated for 10 min at room temperature. Meanwhile the cells were rinsed with 1 ml of warm DMEM-M and 0.5 ml portion of the virus-plant extract mixture was added to duplicate wells. Following incubation of cells with the virus extract mixture for 2.5h at humidified atmosphere comprising 5% CO_2 at 37° C (CO_2 incubator) this inoculum was removed and 1.5 ml of respective plant extract dilution in methyl cellulose medium (methyl cellulose stock diluted 1:2 in DMEM-M) was added to duplicate wells. The cells were then incubated for 3 days in the CO_2 incubator and the devolved viral plaque were stained with crystal violet stock and the viral plaque counted under a microscope. A modification of this assay relying on incubation of cells with plants extracts prior to addition of the virus to cells was performed in a similar manner expect for that the cells were preincubated with 0.5 ml portion of serial 5-fold dilutions of plant extract (ranged from 16 to 50000 μg/ml) for 1h in the CO_2 incubator prior to addition 300 PFU of RSV2.

The viral yield reduction assay was performed as follows. HEp-2 cells seeded at 1.2×10^5 cells/ml were growing for one day in 12 well plates to became ~70% confluent. The cells were rinsed with 1 ml of DMEM-M, and 1 ml of the same medium comprising 400 or 2000 μg/ml of the test plant extract was added. Subsequently the cells were inoculated with RSV-A2 strain in 50 μl of DMEM-M at a multiplicity of infection (MOI) of 0.01 or 1 PFU/cell. Following incubation for 2.5h in the CO_2 incubator, the cells were rinsed twice with 0.5 ml of DMEM-M and 1 ml volumes of fresh extract dilution were added. After incubation of cells for 48h in the CO_2 incubator, the viral infectivity (number of viral PFU/ml) was titrated in the infectious supernatant culture medium (extracellular virus) and in cells (cell-associated virus).

Cell-associated virus was prepared for titration by scraping and vertexing of infected cells prior to the titter determination. The RSV titration was performed as described by Lundin at al. [29], and the viral plagues were counted under the microscope and the viral titter calculated as a PFU/ml.

The time-of-addition assay. HEp-2 cells were seeded in 12 well plates, and in 24 well plates for the virus titration. The cells in 12 well plates were rinsed with 1 ml of DMEM-M and 875 µl of fresh medium was added. Then the cells were infected with 20 µl of RSV A2 stock (MOI =1), incubated for 2h in the CO_2 incubator, rinsed triple with 0.5 ml of the DMEM-M, and then received 0.9 ml of DMEM-M. Then, 100 µl volumes of the plant extract, prediluted in DMEM-M to contain 4000 µg/ml, were added to cells (final concentration 400 µg/ml) at 0h (i.e., just prior to the infection), at 2h (i.e., at the end of 2h period of RSV infection after the cells had been washed) and at 3, 4, 5h post infection. Following incubation of cells for 48h in a CO_2 incubator. The virus that been produced in the supernatant (Extracellular virus) and on the surface of infected cells (Cell-associated virus) was harvested and subjected to the titter (PFU/ml) determination by plaque assay as described above in the virus yield reduction assay.

The virucidal assay. Four hundred and eighty µl of DMEM-M and 20 µl of undiluted virus stock were added to sterile 2-ml vials. The test plant extract at 1000 µg/ml and 2000 µg/ml were added to the respective vial and mixed by gentle pipetting. A control sample containing 480 µl of DMEM-M and 20 µl of undiluted virus stock received 5 µl of redistilled water instead. The mixtures were then incubated for 15 min at 37°C water bath, and their residual infectivity determined by the plaque titration immediately at the end of incubation period. The virus titration was performed as described by Lundin et al. (2013) [29].

The cytotoxicity assay. HEp-2 cells were seeded in 96 well plates, and after one day of culture the supernatant culture fluid was removed, and the cells rinsed once with 100 µl of DMEM_M. Subsequently, 100 µl volumes of the test plant extracts were added at 5-fold increasing concentrations ranging from 16-50000 µg/ml. The cells were then incubated at 37° C for 72h and 20 µl of the prewarmed CellTiter 96 Aquous One solution reagent (Promega) was added. Following incubation of cells for 1-2h at 37°C in CO_2, the optical density was read at wavelength 490 nm against a background of 650 nm using a precision microplate reader.

Statistical analysis. All the data were analyzed using Graphpad Prism. We used unpaired student t-test for analysis of results from the viral yield reduction assay, the time-of-addition assay, and the virucidal assay. *P* values of the threshold ≤ 0.05*; ≤ 0.01**; and ≤ 0.005*** were used to grade a level of statistical significance of the data.

Results

Screening of medical plants for anti-RSV activity. Based on presumed use of certain medical plants in treatment of upper respiratory infections, a collection of seven medical plants and spices was screened for anti-RSV activity in cultures of HEp-2 cells. A water extract of these plants was prepared and tested in viral plaque reduction assay. As seen in Fig. 1 of the seven plants examined only clove exhibited substantial anti-RSV activity reducing the virus-induced plaques by 50% at a concentration of 130 µg/ml. The remaining plants showed no antiviral activity or exhibited toxicity for HEp-2 cells and at the higher concentrations and therefore their anti-RSV activity and these concentrations could not be discernible.
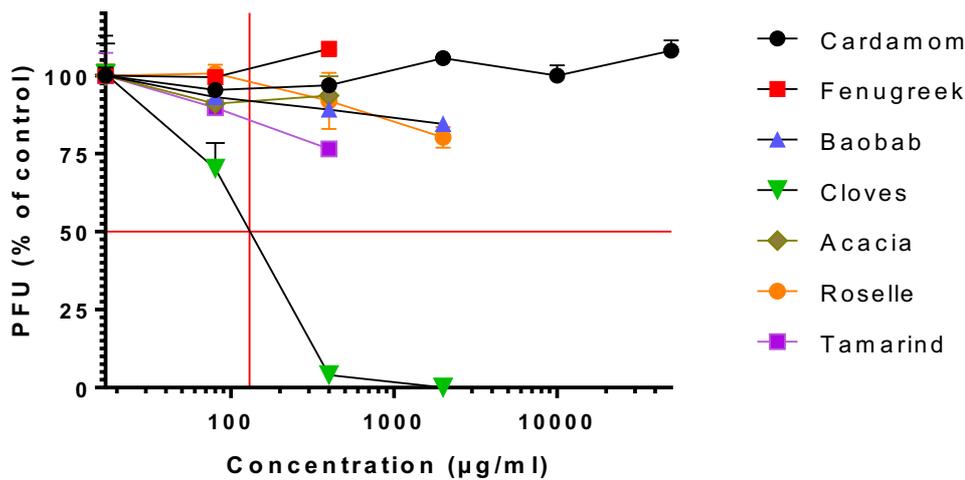
Fig. 1. Screening of medicinal plants and spices collection for anti-RSV activity. Water extracts of specific medicinal plants were mixed with RSV prior addition of the mixture to HEp-2 cells. The plant extracts were examined up to maximum tolerated non-cytotoxic concentrations. The data represent a percentage of a number of viral plaques (Plaque forming units (PFU)) that developed in the presence of plant extract relative to mock treated controls. The results are means of duplicate determination from a single screening experiment. The IC50 value was interpolated from the dose-response curve using GraphPad Prism program version 7.03. two

Anti-RSV potency and toxicity of clove extract for HEp-2 cells. To this end, three different experiments have been performed. The first experiment was done to further investigate the anti-RSV potency of clove extract, comparing by the plaque reduction assay the prior clove-virus preincubation with the prior clove-cell preincubation. Then a viral yield reduction assay was performed to study the amount of virus that been produced in the presence of clove extract, and lastly a cytotoxicity assay was performed to study the toxicity of clove extract for cells and to calculate its selectivity index.

Data in Fig. 2A revealed that clove extract inhibited by 50% the RSV induced plaque formation in HEp-2 cells with IC50 value of 102 μg/ml when the virus was preincubated with extract prior to the addition to cells, and with IC50 value of 152 μg/ml when the extract was preincubated

with cells prior to the addition of virus. These IC50 values were interpolated from the dose response curves in Fig. 2A.
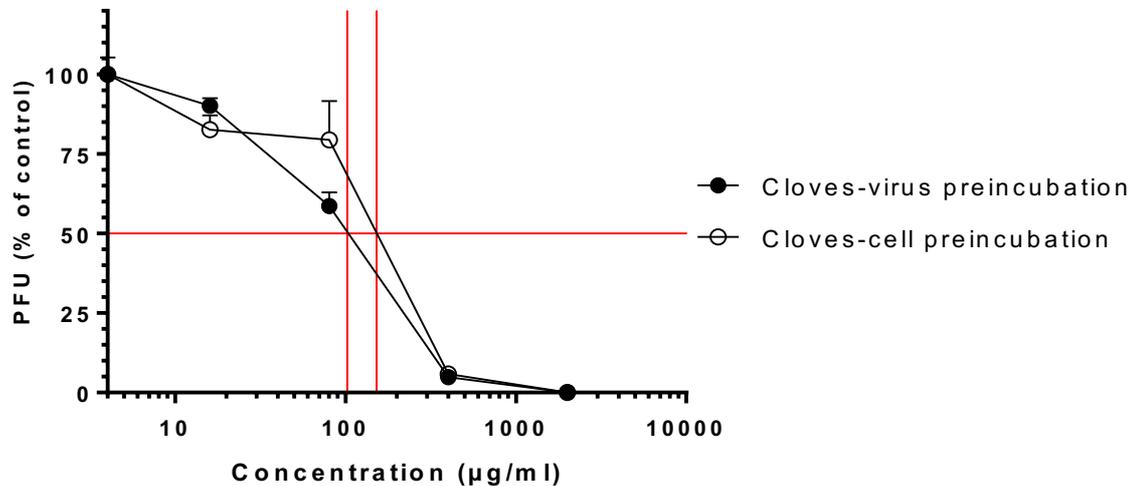

Fig 2.A. Determination of anti-RSV activity of clove extract by the virus plaque reduction assay. Two separate experiments were performed in duplicates, and the results shown are percentages of a number of viral plaques (PFU) found in extract treated virus or cells relative to the mock-treated controls. The IC50 values were interpolated from the dose-response curve using GraphPad Prism program version 7.03.

Efficiently and at relatively low concentrations inhibited RSV infectivity and that the virus particles rather than cells are most likely to be targeted.

The viral yield reduction assay (Fig. 2B) showed that clove extract inhibited production of extracellular virus (EX), i.e., RSV spontaneously released from infected cells to culture medium, and the cell-associated virus (CA), i.e., RSV present on the surface of infected cells and forcibly released from this site by vortexing. The virus titter determination of EX and CA virus revealed that clove extract at 400 µg/ml significantly reduced the production of EX and CA viruses by ~1.5 and 1 $\log_{10}$ respectively. At a concentration of 2000 µg/ml of clove

extract the viral yield reduction was much greater approaching ~4 $\log_{10}$ for EX and CA
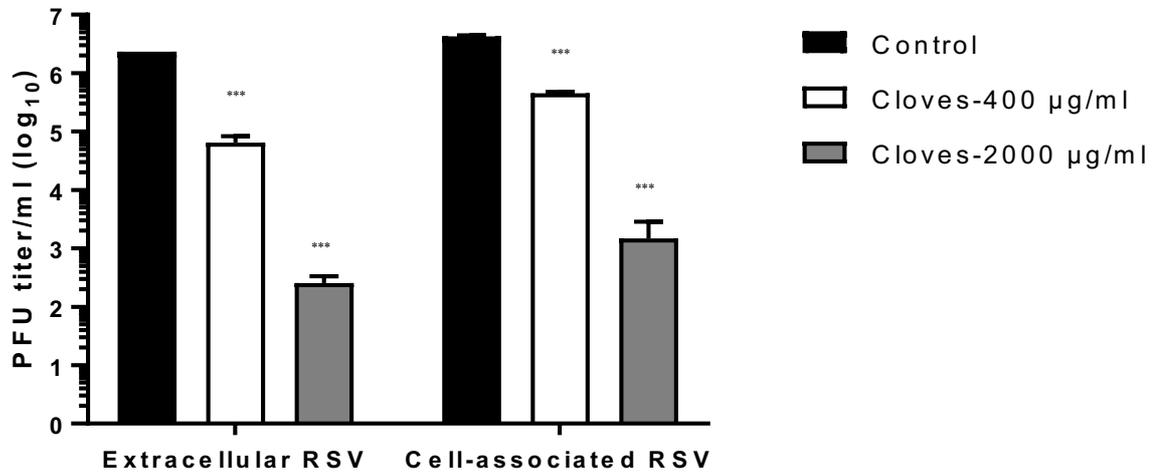
viruses (Fig. 2B).

Fig.2B. The viral yield reduction assay. The RSV yield of extracellular and cell-associated virus produced in the presence of 400 and 2000 μg/ml of clove extract was titrated by the viral plaque forming unit (PFU) assay. Statistically significant differences as related to control at p value <0.005 are denoted with (***).

As seen in Fig.3, the concentration of clove extract that reduced the viability of HEp-2 cells

by 50% (cytotoxicity concentration 50 (CC50)) was 4500 μg/ml. Based on this value, the

selectivity index (SI) (IC50/CC50) of clove extract for RSV infections was calculated

(4500/102) as 44. This SI value of shows that anti-RSV activity of clove extract occurs at

concentration of 44 times lower that the threshold for cytotoxic concentrations.
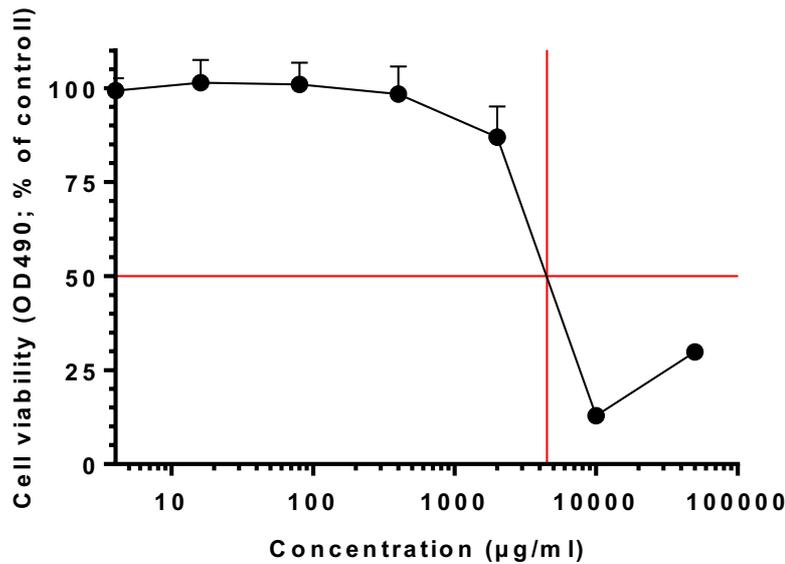
Fig.3. Cytotoxicity of clove extract for HEp-2 cells. HEp-2 cells were incubated with increasing concentrations of clove extract for 3 days, and its cytotoxicity was assayed using tetrazolium-based MTS salt assay. Results are expressed as percentages of OD490 values in extract-treated cells relative to mock-treated cells. Two separate experiments were performed in duplicates. The IC50 value was interpolated from the dose-response curve using GraphPad Prism program version 7.03.

Mechanism of anti-RSV activity of clove extract. This was studied by preforming the time-of-addition assay and the virucidal assay. The time-of-addition assay was preformed to provide initial guidance to determine where in the viral cycle the viral inhibition of clove extract takes place. This was done by adding the clove extract to HEp-2 cells at different time points relative to the virus inoculation. To determinate whether the clove extract directly targeted the free viral particles (virucidal effect) and decreased its infectivity, the virucidal assay was performed.
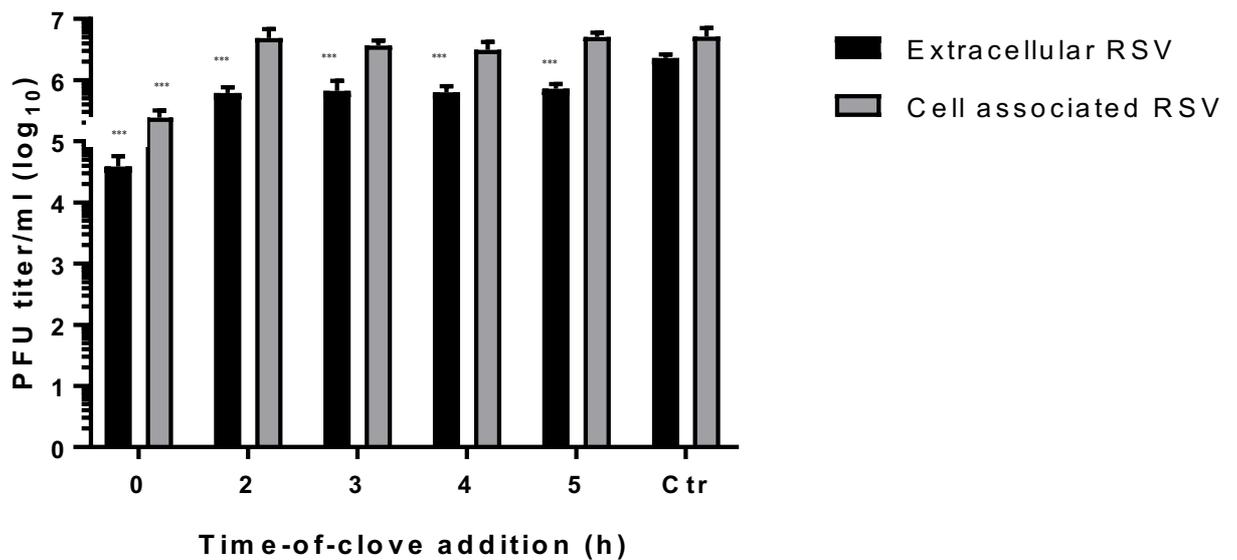
Fig.4 The time of clove addition to infected cells affects RSV yield. Clove extract was added to HEp-2 cells at different time points relative to infection of cells with RSV. The virus infection o cells took place between 0-2 h. Addition of clove extract e.g. at 3 h indicates that it occurred at 3 h relative to the beginning of infection of cells with RSV. The yield of extracellular and cell-associated virus was determined at 48 h after infection of cells. Two separate experiments, each in two technical replicates, were performed, and the results are expressed as a mean viral plaque forming units (PFU) titre. Statistically significant differences as related to the virus titre propagated in the absence of extract (Ctr) are denoted with *** (P <0.005).

As seen in Fig. 4 significant reduction ($\sim$ 1-1.5 $\log_{10}$) of the viral titre of both extracellular and cell-associated virus occurred when the clove extract was added to cells at 0 h, i.e., simultaneously with the virus inoculum at the very beginning of infection of cells. Modest but still significant decrease of the RSV yield observed with extracellular but not cell-associated virus at latter time points post infection could be attributed to the fact that the cells were infected at a multiplicity of infection (MOI) of 1 indicating that statistically not all cells were infected at time 0 h, and the clove extract might have affected the spread of progeny virions

from infected to non-infected cells thus affecting the titre of cell associated virus. Anyhow, these results suggest that clove extract may target the RSV particles yet before their attachment to and infection of cells. To test this possibility a virucidal assay in which the extract was mixed with and then incubated with viral particles.

The virucidal experiment assay revealed that the co-incubation of clove extract with RSV decreased the RSV infectivity by 1 $\log_{10}$ at 2 mg/ml and 3.5 $\log_{10}$ at 10 mg/ml of the extract (Fig. 5). This indicates that clove extract exerts anti-RSV activity by directly targeting the free RSV particles and destroying their infectivity.
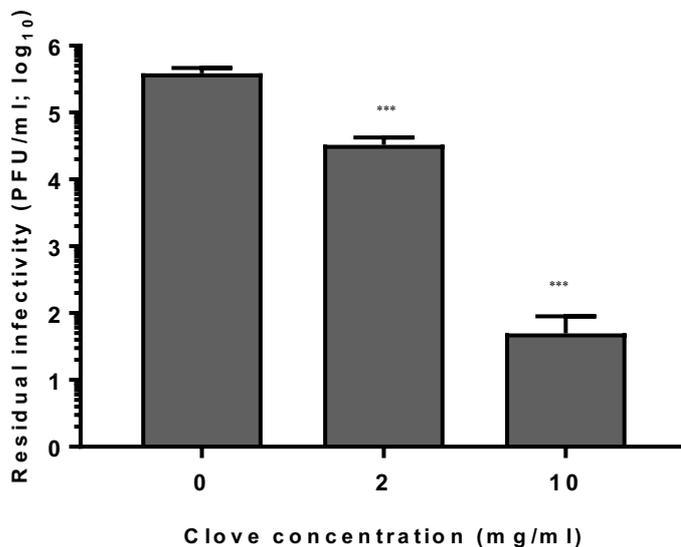
Fig.5. Virucidal activity of clove extract. Approximately 5 x 10^5 plaque forming units (PFU) or RSV was mixed with clove extract at indicated concentrations and incubated for 15 min at 37°C water bath. Four separate experiments, each in two technical replicates, were performed, and the results are expressed as the titre of residual infectivity of clove-treated virus and mock-treated virus control. The results shown are mean infectious titres of all experiment performed. Statistically significant differences at the P value <0.005 are denoted with ***.

Discussion

NP can occasionally result in the development of new drugs, therefore in this study we screened a collection of traditional medicine plants against RSV. The result from the screening showed that of the seven plants examined only clove exhibited substantial anti-RSV activity reducing the number of virus-induced plaques by 50% at a concentration of 130 μg/ml. The remaining plants showed no antiviral activity or exhibited toxicity for HEp-2 cells at the higher concentrations and therefore their anti-RSV activity and these concentrations could not be discerned. However, this study does not exclude the effect of these plants against upper respiratory symptoms or their possible antiviral activity as they may have other mechanism of action i.e. modulating the immune system for better anti-microbial response thus reducing the symptoms of viral infections. These plants may contain for instance high concentration of vitamin C that is scientifically suggested to help against viral infections [31]. According to Rahul et al., (2015) one of the plants examined i.e. baobab contains 10 times higher level of c-vitamin than oranges, a known source of this substance [32]. Another plant screened for anti-RSV activity, i.e., tamarind has been shown to have antibacterial activity [33]. This may explain its use among the Nubian locals in treatment of upper respiratory infections that could be caused by bacteria beside viruses. Roselle was reported to have specific antiviral activity against influenza virus [34]. *Acacia nilotica* according to the locals it is smoked and inhaled in treatment of cough. Acacia has also shown to have antimicrobial

activity [35]. In the present study acacia was used tested in a form of water extract and not a smoke so a question as to whether this alteration has affected antiviral activity cannot be addressed. Kandikattu et al., (2017) found that cardamom extract exhibited anti-inflammatory effect by down-regulating at least 4 inflammatory cytokines [36]. Given these facts, further studies are needed to investigate other possible mechanism of antiviral activity of these plants. In this study, we chose to focus on our hit plant, the clove flower bud specifically because of its substantial anti-RSV activity detected here by us.

To our knowledge this study is the first to evaluate the anti-RSV activity of clove extract. The anti-RSV activity of clove was manifested by a potent inhibition of RSV infection of HEp-2 cells with IC50 value of 102 µg/ml when the virus was preincubated with extract prior to the addition to cells, and with IC50 value of 152 µg/ml when the extract was preincubated with cells prior to the addition of virus, and also by reducing at 2000 µg/ml the viral yield production by ~10000 fold. This potent anti-RSV effect of clove extract indicate that clove produces compound(s) that adversely affect infectivity of microbial pathogens. The fact that the inhibition occurred at lesser concentration when the virus was preincubated with extract as related to preincubation of extract with cells indicates that clove preferentially targets the virus particle rather than the cells and may block or destroy the virus particle infectivity before its attachment to cells.

Furthermore, this activity of clove came with relatively low cytotoxicity and substantial IC50 value which resulted in selectivity index of 44, showing that anti-RSV activity of clove extract occurs at concentration of 44 times lower that the threshold for cytotoxic concentrations pointing towards high selectivity for RSV.

With respect to the mechanism of actionwe found that clove specifically affected the early steps of viral infection of cells occurring prior to the virus attachment to cells, i.e. the clove extract appeared to directly target the free RSV particles, indicating a virucidal (the virus particle-blocking or destroying) activity.

The antimicrobial activity of clove has been previously suggested by some studies. These studies demonstrated strong antibacterial activity of clove extract against several pathogens including *Campylobacter jenjuni*, *Salmonella enteritis, Escherichia coli,* Staphylococcus *aureus and Listeria monoctogenes*. Interestingly this activity relied on bactericidal effect, i.e., on direct targeting of bacteria resulting in inhibition of pathogen infectivity or destroying of bacteria integrity[37-41]. This mode of activity of clove extract is similar to the one observed in the present study where infectivity of RSV particles was inhibited or destroyed (virucidal activity) by clove extract. In addition, clove extract also showed fungicidal and antiviral activity. In particular, Hussein et al. (2000) found that clove extract inhibited replication of hepatitis C virus (HCV) by 90% at 100 μg/ml,[42] i.e., at concentration similar to the IC50 value observed by us in the present study with RSV [42]. Furthermore, Kurokawa et al. (1998) isolated from clove extract compound eugeniin, which exhibited specific inhibition for HSV-1 DNA polymerase activity [43].

Altogether clove extract seems to have a general microbicidal/virucidal properties as demonstrated by some previous studies and our present investigation. However, one cannot exclude a true antiviral mechanism with a specific RSV component targeted, and therefore the present investigation will be extended by HPLC (high-performance liquid chromatography) analysis to identify the active compound in clove extract for further evaluation of the anti-RSV mechanism of action. It is generally accepted that compounds exhibiting virucidal

mode-of-activity are less likely to be used as systemic drugs, but they can be used for local treatment including inhalers or disinfectants [44].

Analytical data presented in some studies show that clove extract composes of 88% eugenol, 5.6% eugenyl acetate, and 34 other compounds in small percentages [43, 45]. Therefor we tested eugoneol and eugenyl acetate for anti-RSV activity. Preliminary data indicated that both these compounds exhibited lesser anti-RSV activity that the whole clove extract (data not shown) suggesting that some other compounds from clove extract could be also involved in anti-RSV activity.

HPLC-based identification of active antiviral ingredient(s) of the extract could lead, after extensive safety and efficacy studies, to development of an air disinfectant, having in mind the fact that in the past clove was used to clean the air from airborne pathogens. Nowadays the most common way for cleaning air from unwanted particles of dust, pollen, mold and allergens is by air purifiers, using filters to trap particles within the air [46]. The concept of air sanitizer is not fully developed as yet. One study performed in 1944 suggested the use of vapor of glycols as air sanitizer since this component caused significant decrease in the number of viable microbes in the air [47]. Our results suggest that clove component(s) can be considered a likely candidate for studies on prevention the spreading of community acquired virus infection.

Methodological consideration and study limitation
In this study we had some limitation. These mainly have to do with, contamination and toxicity. The intrinsic toxicity and mold contamination affected the count of viral plaque or it made it impossible for the virus to infect such cells.

We used viral plaque reduction assay to search for possible anti-viral activity present in plant extracts in RSV susceptible HEp-2 cells and protection of cells against RSV, i.e., lack of development of RSV induced syndical of cells was considered as hit. The clove extract fulfilled this requirement. At the same time the plants extract was examined for cytotoxicity to make sure that antiviral effect of the extract is viral specific and not due to adverse, toxic effects on cells. One limitation of this approach is that the hit compounds occurring in the plant extract at relatively low concentration i.e., below the threshold for cytotoxicity are difficult to be detected. Given that fractionation of the extract using preparative HPLC may solve the problem. Another limitation of this study is the presence of mold contamination in same but not all extracts. This contamination seems to be intrinsic because it concerns only some extract but anyhow did not all examination for anti-RSV activity at high concentrations. Using anti-fungal antibiotic amphotericn B could eastly solved the problem.

Conclusions

NP are valuable library in screening for novel drugs. In this study we found that Clove extract showed definite anti-RSV activity. This activity relied on direct targeting of free virus particles thus destroying their infectivity. Such mechanism of anti-RSV activity of clove extract permit it use for local treatment or as air disinfectant.

Populärvetenskaplig sammanfattning på svenska

Naturliga produkter i form av bland annat örtmediciner har använts av människor i tusentals år för medicinska ändamål. Denna användning har ibland resulterat i utveckling av nya läkemedel med vetenskapligt dokumenterad effekt. Ett välkänt exempel på ett sådant läkemedel är antibiotikan penicillin som produceras av mögelsläktet penicillium. Ett annat exempel är malariamedicinen artemisinin, som extraheras från sommarmalört.

Respiratoriskt syncytiellt virus eller RS-virus som det också kallas, är ett luftvägsvirus som är känt för att orsaka svåra luftvägsinfektioner hos nyfödda och äldre. Hos friska vuxna orsakar RS-virus lindriga förkylningssymtom eller verkar icke symptomgivande. Viruset sprider sig genom luft, droppar och genom direktkontakt med smittade vätskor.

Idag finns det varken något registrerat läkemedel tillgängligt mot detta viktiga virus, eller något vaccin. Bristen på effektiv antiviral terapi gör att behandling av akut RS-virusinfektion idag huvudsakligen omfattar stödjande vård.

Syftet med detta examensarbete var att undersöka om extraherade prover från en samling av traditionella medicinska växter uppvisar antiviral aktivitet (hämning) mot RS-virus i cellkultur.

En samling traditionella örtmediciner från Sudan pulveriserades och extraherades med sterilt vatten för att sedan undersökas för antiviral aktivitet mot RS-virus. Nejlike-extrakt visade en stark hämning av viruset, och studerades därför mer i detalj. Avsikten var att bestämma dess potens avseende hämningen av viruset, toxicitet (giftighet) för humana celler och att undersöka dess verkningsmekanism.

Av flera undersökta växter visade endast Nejlike-extrakt antiviral aktivitet mot RS-virus i cellkultur vilket visade sig genom en markant minskning av virus-produktion i de infekterade cellerna. Aktiviteten var dessutom mycket specifik för just RS-virus och verkade rikta sig direkt mot fria RSV-partiklar under ett tidigt steg av virusinfektionen. Dessutom var toxiciteten mot cellerna låg.

Vår slutsats är att Nejlike-extrakt visade en potent aktivitet mot RS-virus som verkade vara virus-dödande (virucidal). Ytterligare uppföljning av denna studie, inkluderande identifiering av dess aktiva beståndsdelar, krävs inför en eventuell utveckling av nejlike-extraktet antingen som läkemedel eller som luftdesinfektionsmedel mot RS-virus.

Acknowledgments

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