Phytomedicine 19 (2012) 231–235

Contents lists available at SciVerse ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

In vitro anti-viral effect of β-santalol against influenza viral replication

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ARTICLE INFO

Keywords:
β-Santalol
Oseltamivir
RT-PCR
Antiviral
Influenza

ABSTRACT

The anti-influenza A/HK (H3N2) virus activity of β-santalol was evaluated in MDCK cells and investigated the effect of β-santalol on synthesis of viral mRNAs. β-Santalol was investigated for its antiviral activity against influenza A/HK (H3N2) virus using a cytopathic effect (CPE) reduction method. β-Santalol exhibited anti-influenza A/HK (H3N2) virus activity of 86% with no cytotoxicity at the concentration of 100 μg/ml reducing the formation of a visible CPE. Oseltamivir also showed moderate antiviral activity of about 83% against influenza A/HK (H3N2) virus at the concentration of 100 μg/ml. Furthermore, the mechanism of anti-influenza virus action in the inhibition of viral mRNA synthesis was analyzed by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), and the data indicated an inhibitory effect in late viral RNA synthesis compared with oseltamivir in the presence of 100 μg/ml of β-santalol. β-Santalol should be further studied for therapeutic and prophylactic potential especially for influenza epidemics and pandemics.

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Introduction

Influenza virus is documented to cause epidemics and pandemics in human population over several centuries. Influenza A virus has several zoonite hosts, therefore cannot be eradicated from human populations. Despite widespread access to vaccines and antiviral therapies, influenza continues to be a major cause of morbidity and mortality. About 31,000 deaths each year in the US are associated with influenza infections. Influenza A viruses are respiratory pathogens that can raise to severe illness in humans. In the 20th century, three influenza pandemics occurred: the H1N1 Spanish Flu in 1918, the H2N2 Asian Flu in 1957 and the H3N2 Hong Kong Flu in 1968 (Kilbourne 2006). In developing countries the availability of modern medicines is limited in supply. Therefore the traditional medicine is still the mainstay of health care and most drugs come from plants. Thus the search on new drugs is still continued and natural products from plants are kept on emerging in recent past years. Few substances are known to be effective against viral infections *in vivo* (Balfour 1999).

Currently, two classes of anti-influenza agents have been reported for influenza management and are under consideration for stockpiling in the event of an influenza pandemic; one of which targets the M2 ion channels (e.g., amantadine and rimantadine) and the another one inhibits neuraminidase (e.g., oseltamivir and zanamivir). In fact, influenza A viruses still pose a major burden to human health and cannot be completely eradicated due to their large natural reservoir. Treatment with amantadine and their derivatives rapidly results in the emergence of resistant variants and hence is not recommended for the general and uncontrolled use (Hayden and Hay 1992). Oseltamivir is considered the drug of choice for patients with pandemic influenza for whom drug treatment is recommended because adamantanes seem to be ineffective against pandemic A/H1N1 influenza virus and zanamivir (the other available neuraminidase inhibitor) is contraindicated in people with underlying respiratory conditions and difficult to administer in younger children (Garman and Laver 2004). Emergence of resistance to oseltamivir in human influenza A viruses (Ison et al. 2006) and the H5N1 subtype in Vietnam is a cause for concern. However, resistance has not been reported for the other neuraminidase inhibitor, zanamivir. Nevertheless, expanding the range of antiviral drugs that effectively inhibit replications of the influenza A virus, or potentially act in synergy with neuraminidase inhibitors is a matter of urgency.

Santalol is a fragrance ingredient used in decorative cosmetics, fine fragrances, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents. Its use worldwide is in the region of 0.1–1 metric tonnes per annum (Bhatia et al. 2008). The essential oil emulsion or paste of sandalwood is routinely used in India as an ayurvedic medicine to inflammatory and eruptive skin diseases. Sandalwood oil treatment significantly decreased papilloma incidence by 67%, multiplicity by 96% and TPA-induced ODC activity by 70%. This oil could also

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doi:10.1016/j.phymed.2011.11.006

be used as an effective chemo preventive agent against skin cancer (Dwivedi and Ghazaleh 1997). The bioactive compounds of sandalwood such as (Z)-beta-santalol, (Z)-lanceol were isolated from *Santalum album* have strong anti-*H. pylori* activities against a clarithromycin-resistant strain (Ochi et al. 2005). It is reported that Sandalwood oil has inhibitory properties against Herpes simplex viruses-1 and -2 (Benencia and Courreges 1999). The use of Santalol has been reported in murine models of skin carcinogenesis employing human epi-dermoid carcinoma A-431 cells (Scartezzini and Speroni 2000). No data available with reference to mutagenicity, genotoxicity and carcinogenicity of β-santalol. The potential effect of β-santalol on the replication of influenza virus has not yet been systemically studied and limited information is available on this context. In this study, we examined the antiviral activity of β-santalol against influenza A/HK (H3N2) virus and also attempted to elucidate the effects of β-santalol on influenza A/HK (H3N2) virus replication in MDCK cell line.

Materials and methods

Plant material

Chips of dried sandalwood (*Santalum album* L.) were purchased from a local market in India. One of the authors (Hideo Yamada, Yamada-matsu Co., Ltd.) imported the material, which did not contain significant amounts of moisture.

Hexane extraction of sandalwood chips

Chips of sandalwood (30–100 g) were extracted with hexane (300–1000 ml) at room temperature. Removal of the solvent by rotary evaporation at room temperature afforded reddish brown oil in 7.1% yield. β-Santalol from hexane extract of sandalwood was isolated from fractions separated by distillation and subsequent chromatography.

High Pressure Liquid Chromatography (HPLC) analysis

All solvents used were of analytical chromatographic grade (Carlo Erba reagents). HPLC was performed with a HP Ti series 1050 liquid chromatograph, equipped with a photodiode array detector (DAD, HPseries 1050). Solutions of the tested oil (5% v/v in *n*-hexane or ethanol) was subjected to normal phase HPLC analysis carried out on a Phenomenex Hypersil 5 CN (5 μm, 25 mm × 4.6 mm) column using a mobile phase of *n*-hexane at a flow rate of 1.0 ml/min. The injector was a Rheodyne model valve with a 20-μl loop. UV detection (DAD) at two wavelengths (245 and 265 nm) was recorded. Eluate fractions obtained from HPLC analyses (*n*-hexane) were further subjected to GC–MS analysis after concentration under vacuum.

Gas Chromatography–Mass Spectrometry (GC–MS) analysis

The chemical composition of the essential oil was analyzed using GC–MS. The essential oil (10 μl) was dissolved in acetone (100 μl) and 1 μl of the solution was injected into a GC–MS (QP-2010, Shimadzu Co., Kyoto, Japan). The capillary column was Rtx-5MS (length = 30 m, i.d. = 0.25 mm, thickness = 0.25 μm). Helium was used as the carrier gas at a flow rate of 0.94 ml/min. The column inlet pressure was 55.8 kPa. The GC column oven temperature was increased from 60 to 170 °C at a rate of 10 °C/min, with a final hold time of 10 min. Injector and detector temperatures were maintained at 150 °C. EI mode was at 70 eV, while mass spectra were recorded in the 45–450 amu range and ion source-temperature was 200 °C. Essential oil components were quantified by relative percent peak area of TIC from the MS signal and identified by comparing their mass fragmentation pattern (Tian et al. 2011).

Viruses, cells and reagents

Influenza A/HK (H3N2) virus obtained from King Institute of Preventive Medicine & Research, Virology Department, Chennai. Virus was propagated in Madin-Darby canine kidney (MDCK) cells at 37 °C. MDCK cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic–antimycotic solution. Antibiotic–antimycotic solution, trypsin–EDTA, FBS and MEM were supplied by Himedia. MDCK Cell lines were purchased from National Center for Cell Sciences (NCCS, Pune). All other chemicals were of reagent grade. Oseltamivir (Sigma Aldrich) Stock solutions (10 mg/ml) of the antiviral compounds were made in dimethyl sulfoxide (DMSO) and were subsequently diluted in appropriate culture media. The final DMSO concentration was a maximum of 0.1%, which had no effect on the cell cultures. Therefore, 0.1% DMSO was also added to all no-drug control samples.

Assays of antiviral activity and cytotoxicity

Assays of antiviral activity and cytotoxicity were evaluated by the SRB method using cytopathic effect (CPE) reduction recently reported (Choi et al. 2009a,b). Oseltamivir was used as positive, and DMSO was used as negative control. The effect of β-santalol on influenza virus-induced CPE was observed. Briefly, MDCK cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells per well. Next day, medium was removed and washed with PBS. Then, 0.09 ml of diluted virus suspension and 0.01 ml of medium supplemented with trypsin–EDTA containing β-santalol of 100 μg/ml were added. After incubation at 37 °C in 5% CO_2 for 2 days, the morphology of cells was observed under microscope of 32 × 10 magnifications (Lobamed, Germany), and images were recorded.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis

MDCK cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells per well. After 24 h, medium was removed and the cells were washed with PBS. Subsequently, 0.09 ml of diluted virus suspension and 0.01 ml of medium supplemented with trypsin–EDTA containing β-santalol or oseltamivir of 100 μg/ml were added. After incubation at 37 °C in 5% CO_2 for 48 h, the next step was performed. Total RNA was extracted from cells as described elsewhere (Chomczynski and Sacchi 1987). The parallel expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was tested under the same PCR conditions as an internal standard. Randomly primed cDNA obtained by reverse transcription (RT)-PCR was amplified (Rochi, Germany) in a PCR mixture (50 μl) that contained membrane (M) or GAPDHgene primers: M gene, 5′-AGTGAGCGAGGACTGCAGCGT-3′ and 5′-TAGCYTTAGCYGTRGTGCTGGC-3′; GAPDH, 5′-CCCATCACCATCTTCCAGGAGC-3′ and 5′-CCAGTGAGCTTCCCTTCAGC-3′. The products were electrophoresed and visualized by ethidium bromide staining.

Results and discussion

Essential oils analysis

The GC–MS analyses of the extracted sandal wood oil sample contained higher concentration of β-santalol (Fig. 1a), compound and was identified using their mass spectra and retention indices

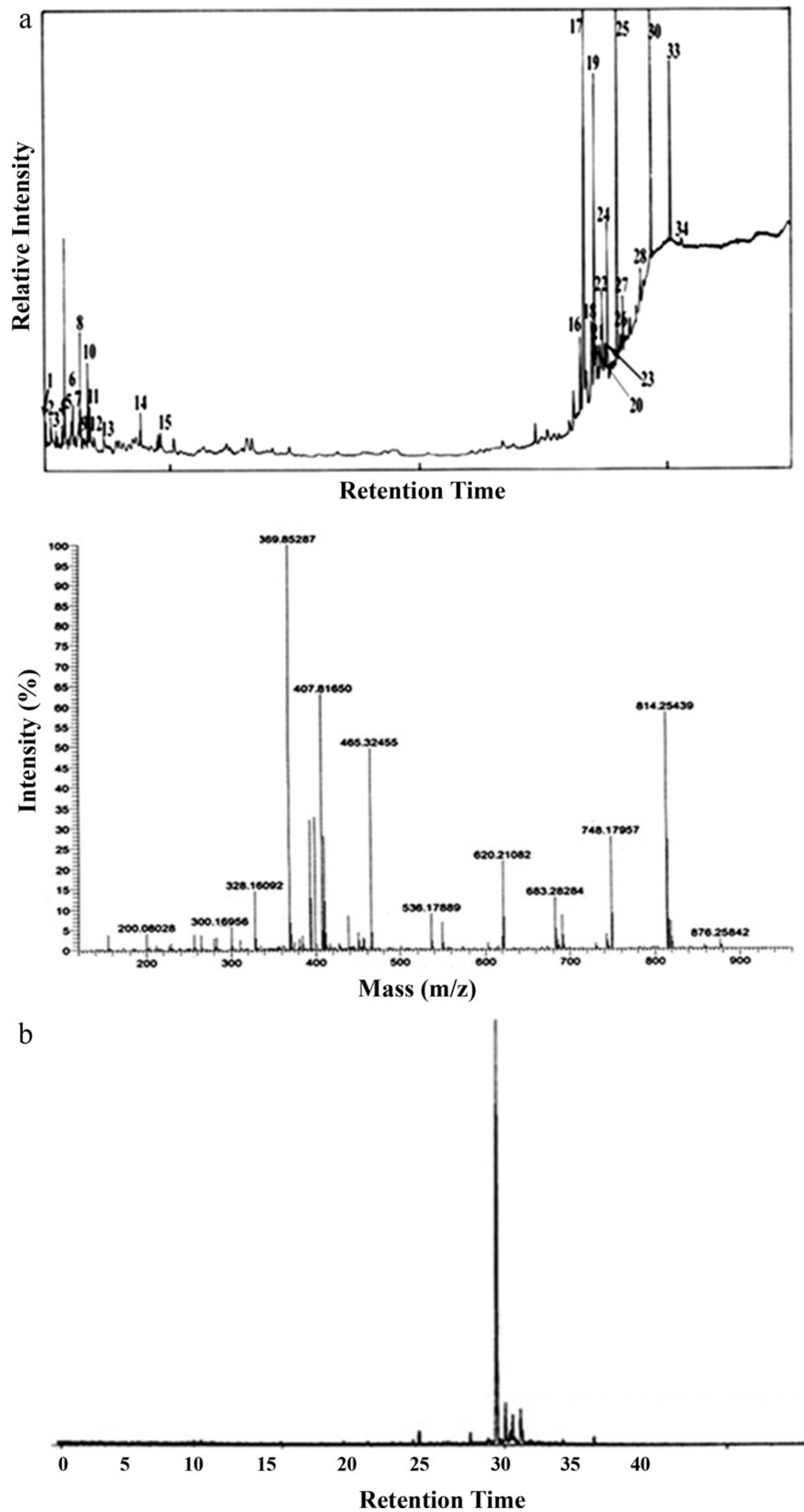

Fig. 1. (a) GC–MS chromatograms of sandal wood essential oil, for chromatographic conditions see experimental section. (b) Purification of β-santalol with the assistance of HPLC. For chromatographic conditions see Experimental section.

(RI) by comparing with the standard. In all of the triplicate samples, β-santalol was found to be the main component that corresponded to about 80% of the oil content with minor fractions of other constituents of oil. The minor fractions were reported as oil was found to contain also sesquiterpene hydrocarbons (ca. 20%, mainly α-co-paene, germacrene D and α-caryophyllene), alcohols (ca. 12%, mainly linalool and α-terpineol) and monoterpene hydrocarbons (ca. 7.5%, mainly myrcene, limonene and the two ocimene isomers), in addition to minor fractions of oxides, and or unidentified structures.

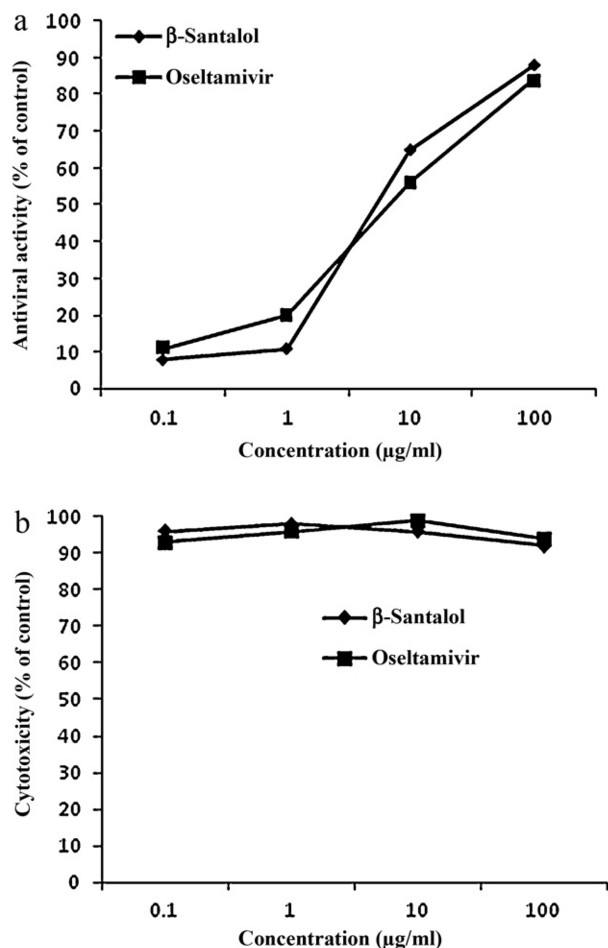

Fig. 2. Antiviral activity of β-santalol against influenza A/HK (H3N2) in MDCK cells. Culture medium was removed from 96-well tissue culture plates and the cells were washed with PBS. Then 0.09 ml of diluted virus suspension and 0.01 ml of medium containing β-santalol or oseltamivir of with a 10-fold diluted concentration ranging from 0.1 to 100 μg/ml were added. After incubation at 37 °C in 5% CO_2 for 2 days, the antiviral activity was investigated by CPE reduction assay. Results are presented as the mean percentage values obtained from the independent experiments carried out in triplicate ±S.D.

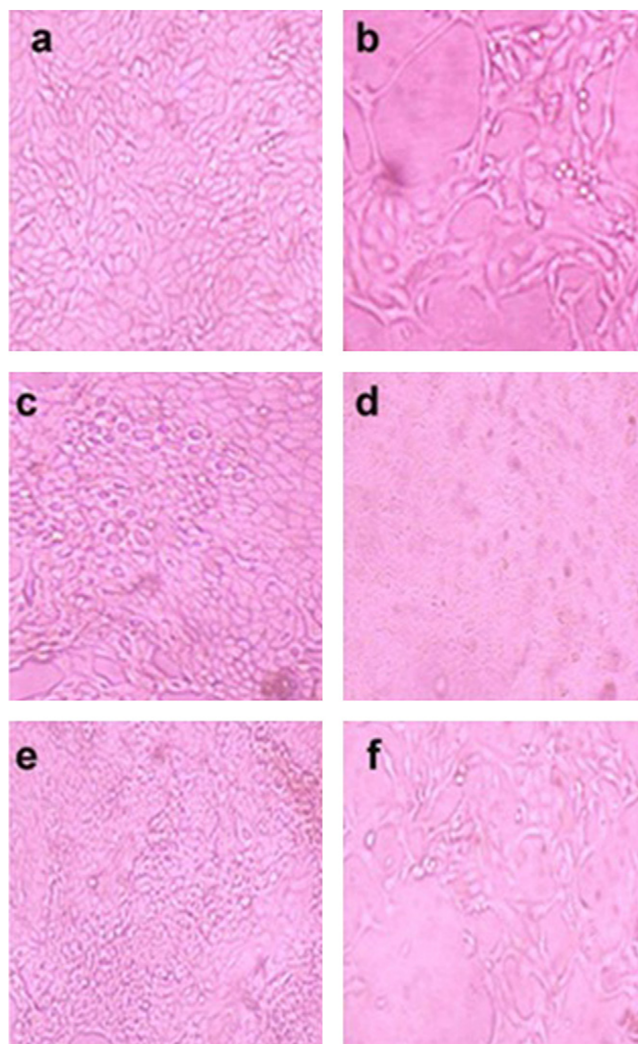

Fig. 3. The effect of β-santalol on influenza virus-induced CPE. The virus-infected cells were treated with oseltamivir or β-santalol of 100 μg/ml. After incubation at 37 °C in 5% CO_2 for 2 days, inhibition of virus replication was evaluated by SRB assay, and the morphology of cells was photographed under a microscope. (A) Non-infected cells; (B) non-infected cells treated with β-santalol; (C) non-infected cells treated with oseltamivir; (D) influenza virus-infected cells with out β-santalol; (E) influenza virus-infected cells with β-santalol; and (F) influenza virusinfected cells with oseltamivir.

The HPLC analyses under normal phase conditions (CN-column and *n*-hexane as mobile phase) were allowed the oil components to separate into different groups of chemical classes of increasing polarity (mainly hydrocarbons, ethers, esters and alcohols). The composition of the HPLC-fractions was subsequently confirmed again by GC–MS analysis. Referring to the HPLC chromatogram (Fig. 1b), the highest intense was found by the GC–MS runs to include almost exclusively sesquiterpene (β-santalol) and its derivatives of the oil.

Antiviral activity of β-santalol against influenza A/HK (H3N2) virus

β-Santalol was investigated for its anti viral activity against influenza A/HK (H3N2) virus and MCDK cell viability. The antiviral assays demonstrated that β-santalol possessed strong antiviral activity of about 86% against influenza A/HK (H3N2) virus at the concentration of 100 μg/ml and anti viral activity of about 40% at the same virus at the concentration 10 μg/ml (Fig. 1). Oseltamivir also did show moderate anti viral activity of about 83% against influenza A/HK (H3N2) virus at the concentration of 100 μg/ml and weak anti viral activity of less than 37% at under of 10 μg/ml (Fig. 2). β-Santalol and oseltamivir were not toxic to MDCK cells with cell viability of about 93% at the concentration of 100 μg/ml (Fig. 2).

The effect of β-santalol on influenza A/HK (H3N2) virus-induced CPE

After 2 day infections of MDCK cells with influenza A/HK (H3N2) virus, there was no difference between the mock cells (Fig. 3A) or cells treated with 100 μg/ml β-santalol (Fig. 3C) or oseltamivir (Fig. 3E) in terms of typical spread-out shapes and normal morphology. The cell proliferation was not significantly affected under the 100 μg/ml concentrations of β-santalol. Infection with influenza A/HK (H3N2) virus in the absence of β-santalol resulted in a severe CPE (Fig. 3B). The addition of β-santalol on infected MDCK cells inhibited the formation of a visible CPE (Fig. 3D). However, the addition of oseltamivir in influenza A/HK (H3N2) virus infected MDCK cell was weakly prevented CPE (Fig. 3F). Thus, this result showed that the CPE of the virus infection was prevented by above mentioned concentrations of β-santalol.

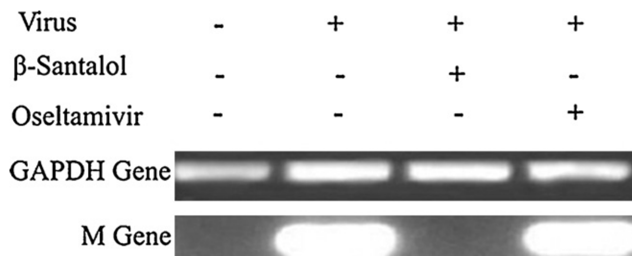

Fig. 4. RT-PCR analysis. Replication of influenza virus from MDCK cells before and at 48 h after infection by influenza virus in the presence of β-santalol (100 μg/ml) or oseltamivir (100 μg/ml) or vehicle alone (control, 0.1% DMSO), as detected by RT-PCR.

Effect of β-santalol on synthesis of viral mRNAs

The viral mRNA synthesis assessed by PCR analysis of the M gene were inhibited completely at β-santalol 100 μg/ml after 48 h infection, while oseltamivir (100 μg/ml) exhibited a small amount of the viral mRNA synthesis product band at 48 h after infection (Fig. 4). The amplification of housekeeping gene GAPDH from the same sample was positive in the PCR analyses (Fig. 4). These findings indicate that β-santalol does decrease viral mRNA synthesis. The development of new antiviral agents for influenza is receiving much greater attention because of the frequent emergence of antiviral resistance during oseltamivir, its association with clinical failure in immuno-compromised hosts, and the emergence of new pandemic subtypes of influenza A virus (Beigel and Bray 2008; Hayden 2001; Ison et al. 2006). The present study describes the cytotoxicity and antiviral activity of β-santalol. β-Santalol was shown to exhibit anti-influenza virus activity against influenza A/HK (H3N2) virus reducing the formation of a visible CPE. These results are similar to the effects of quercetin 3-rhamnoside on influenza virus (Choi et al. 2009a,b). In an *in vitro* study (Benencia and Courreges 1999) tested sandalwood oil for antiviral activity against Herpes simplex viruses-1 and -2. In the presence of sandalwood oil the replication of these viruses was inhibited in a dose-dependent manner. At higher multiplicity of infections, a slight diminution of the effect was noted. Sandalwood oil and its major constituent, α-santolol, have low acute oral and dermal toxicity in laboratory animals. Sandalwood oil was not mutagenic in spore *Rec* assay. Sandalwood oil was found to have anticarcinogenic, antiviral and bactericidal activity. Few cases of irritation or sensitization reactions to sandalwood oil in humans are reported in the literature, although the available information on toxicity of sandalwood oil is limited for evaluation of its safety (Yu et al. 2007; Burdock and Carabin 2008). In conclusion β-santalol is a mixture of small molecules that can efficiently inhibit influenza A/HK (H3N2) virus replication. Therefore, β-santalol is an alternative to agents for treating influenza virus infections. Sandalwood and its oil have a long history of use without any reported adverse effects; therefore consumption of sandalwood oil as an added food ingredient is considered safe at present use levels. Their potent anti-influenza virus activity *in vitro* warrants further studies to evaluate whether β-santalol treatments can also result in anti viral activity *in vivo*.

Acknowledgments

The authors would like to thank Staffs of King Institute of Preventive Medicine & Research, Chennai for providing the all necessary facility to carry out this work.

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