Journal of Applied Pharmaceutical Science

Available online at www.japsonline.com

ISSN: 2231-3354
Received on: 20-06-2012
Revised on: 25-06-2012
Accepted on: 07-07-2012
DOI: 10.7324/JAPS.2012.2734

Anti-Influenza Virus Activities of Commercial Oregano oils and their Carriers

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ABSTRACT

Commercial Oregano oils with high concentrations of carvacrol have been vigorously promoted as antiviral agents effective against colds and 'flu, including the pandemic H1N1 virus. However there seems to be no evidence to support these claims. Furthermore, since carvacrol itself is known to be toxic, so-called "carrier oils", such as olive oil, have been included in formulations to ameliorate the potential toxic effects. We compared the anti-influenza virus activity of several preparations, with and without "carriers", and pure olive oil and carvacrol, by means of quantitative assays for H1N1 influenza virus, and for cytotoxicity in human lung epithelial cells. A range of concentrations was evaluated, including those relevant to consumer applications. All five Oregano oils showed significant antiviral activity, as did olive oil by itself, although their potencies were not comparable to a standardized preparation of *Echinacea purpurea.* Carvacrol was also very active, but it was also strongly cytotoxic. In addition all the Oregano oils were more cytotoxic than *Echinacea purpurea.* Thus certain commercial Oregano oils do possess anti-influenza virus activities, although these are less than a potent standardized *Echinacea* preparation, and furthermore the toxicity of the oils to lung epithelial cells, at doses relevant to consumer applications, is a limiting factor in their usefulness for oral applications.

Keywords: Oregano oil; olive oil; anti-influenza virus; carvacrol; *Echinacea purpurea;* antiviral assays; cytotoxicity

INTRODUCTION

Consumers often choose herbal medicines by consulting sources of information on the internet, or in popular magazines. This is particularly so for products that are alleged to be beneficial in the control of colds and 'flu. However, although there might be considerable anecdotal evidence to support the claims of efficacy in some cases, such advice is not necessarily based on sound scientific evidence.

An example is the current popularity of "Oregano oils", especially those rich in carvacrol, which are promoted as effective oral treatments for the prevention and control of pandemic influenza virus (influenza A virus H1N1, swine-origin influenza virus SO-IV), as well as other respiratory viruses. But experimental evidence supporting these claims is lacking. Furthermore not all commercial Oregano oils are derived from the same species, and in many cases they are diluted with so-called "carrier oils", such as olive oil, presumed to be biologically inert, in order to alleviate the potential cytotoxicity attributed to carvacrol. All of these claims need to be evaluated.

Traditionally various parts of the Oregano plant (*Origanum* spp.) have been used in Mediterranean countries as food spices and preservatives, and as medicines to treat different diseases (Liolios *et al*., 2010). In common with many other plant oils (Bakkali *et al*., 2008), the essential oils of several species of Oregano have been shown in recent studies to possess anti-bacterial activities, and in some cases these activities were attributed to the dominant terpene component carvacrol ((Burt 2004, Sokovic *et al*., 2010, de Souza *et al*., 2010). In addition antiviral activities have been reported for some unrelated essential oils (Carson *et al*., 2006, Cermelli *et al*., 2008, Alim *et al*., 2009), although Sokmen *et al* (2004) did not observe anti-influenza virus activity in their studies of various extracts and oil derived from *Origanum vulgare*. In view of these uncertainties about the feasibility of using Oregano oils as antiviral agents, we decided to investigate several commercial preparations of "Oregano oil", with and without carrier oil (olive oil), for activity against influenza virus H1N1, by means of quantitative antiviral protocols developed in our laboratory for the evaluation of plant extracts. We compared these preparations with pure carvacrol and olive oil, and a known very potent anti-influenza virus extract derived from *Echinacea purpurea* as a reference. Relative cytotoxicities of the oils were also evaluated in a line of human lung epithelial cells. Cytotoxicities were measured for short exposures of 10 minutes, to reflect consumer applications, in addition to the normal 24 hour exposures.

MATERIALS AND METHODS

Test materials

Oregano oils; four popular preparations of "carvacrol-rich" oils were purchased from local suppliers. Three of them had been diluted 3-4 fold with virgin olive oil as "carrier" to mitigate the toxic effects of carvacrol (labeled in this report as ORE 1-3), and one was pure 100% *O. vulgare* without carrier (ORE 4). In addition we tested a product obtained from a popular herbal company which was labeled as a pure carrier free "Oregano-oil" derived from *Thymus capitatus* (ORE 5). These data are summarized in Table 1. Carvacrol was purchased from Sigma-Aldrich, and "pure organic virgin olive oil" and organic canola oil were purchased locally.

Table. 1: Oregano oils tested

Abbreviation	Species[1] (all members of the Family Lamiaceae)	Carvacrol content before blending[1]	Carrier Ratio, ORE:olive oil
ORE 1	*Origanum minutiflorum*	75-85%	25:75 olive oil
ORE 2	*Origanum minutiflorum*	93%	18:82 olive oil
ORE 3	*Origanum vulgare*	80%	40:60 olive oil
ORE 4	*Origanum vulgare*	Not stated	none
ORE 5	*Thymus capitatus*	Not stated	none

According to information supplied on labels and web-sites

Echinacea (EP, Bioforce AG, Roggwil, Switzerland) was a standardized preparation derived by ethanol extraction of freshly harvested *Echinacea purpurea* herb and roots (95:5). The composition of marker compounds (i.e. those compounds known to characterize this species of *Echinacea*) was described previously (Sharma *et al.,* 2009). The concentration of ethanol was 65% v/v but in the experimental reactions and cultures the final concentration of ethanol was too low to cause adverse effects on the cells or viruses.

Cells and virus

MDCK canine kidney cells and A549 human lung epithelial cells were acquired originally from ATCC (American Type Culture Collection, Rockville, MD), and were passaged in Dulbecco MEM (DMEM), in cell culture flasks, supplemented with 5% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere (cell culture reagents were obtained from Invitrogen, Ontario CA). No antibiotics or anti-mycotic agents were used. Influenza virus type A, strain H1N1 (A1/Denver/1/57) was acquired from BC Centre for Disease Control, Vancouver, and was grown and assayed, by plaque formation, in MDCK cells, the standard cell line for growth and measurement of Influenza viruses (WHO manual, 2011).

Antiviral assays

The assay technique was based on our standard techniques for the evaluation of plant extracts for antiviral activity (Vimalanathan *et al*., 2005, Pleschka *et al.,* 2009). The experimental procedure consisted of incubating two-fold dilutions of the test oil or compound in phosphate buffered saline, in 96-well trays, with a known amount of the virus (either 10^3 or 10^5 pfu, as indicated) for 60 min at 22ºC (in triplicate reactions). The reactions were then assayed for residual infectious virus (plaques) in monolayers of freshly confluent MDCK cells, in 6-well culture trays. Reduction in the number of virus plaques represents the degree of antiviral activity. In some experiments, indicated in results, the test agent was incubated with a larger amount of virus (10^5 pfu), and the reaction mixtures were then diluted 50-100 fold followed by plaque assay. Controls consisted of virus in medium without test compound.

Cytotoxicity assay

The Cell Proliferation Assay Kit (XTT) (ATCC, Manassas, VA, USA) was used according to the manufacturer's instructions. The assay detects the reduction of XTT (sodium 2,3,-bis (2- methoxy -4-nitro- 5-sulfophenyl) -5-[(phenylamino)-carbonyl] -2H-tetrazolium inner salt) by mitochondrial dehydrogenase to orange formazan product, which reflects the normal functioning of mitochondrial and cell viability. The amount of the formazan produced is proportional to the number of viable cells (ATCC Manassas, VA, USA).

Human lung epithelial cells (A549 cell line) were used as the indicator cells. The cells (5×10^3) were seeded in each well containing 100µl of the MEM medium supplemented with 5% FBS in a 96-well plate. Cells were grown for 48 h and the test materials, prepared as a series of two-fold dilutions in MEM, without phenol red, were added to the cells and incubated for 10 min (short exposure), followed by removal of test material and incubation for a further 24 h in MEM, or for 24 h (long exposure), followed by assays for cell viability. The results were measured as absorbance at 490 nm in a plate reader, in comparison with similar cells

exposed to medium only. Cytotoxicity is expressed as the concentration of test samples inhibiting cell growth by 50% (IC_{50}). All tests were run in triplicate and mean values recorded.

RESULTS

Test Materials

Table 1 summarizes the relevant characteristics of the test Oregano oils (ORE 1 - ORE 5). ORE 1-3 were representative commercial Oregano oils (*Origanum spp*) diluted with so-called carrier olive oil. ORE 4 was also *O. vulgare* but contained no carrier, and ORE 5 was derived from *Thymus capitatus* although it was labeled as an Oregano oil.

Antiviral activity

Initially each oil with carrier olive oil (ORE 1-3) was tested for anti-influenza virus activity in comparison with the reference Echinacea (EP) and carvacrol. Figure 1 shows the relative inhibitory activity of each serial dilution. All three oils were fully active (i.e. reduced the virus titer by 100%) at 100µl/ml concentration, but their activities decreased at successively higher dilutions until 3.13 µl/ml, when they were no longer antiviral. In contrast EP showed complete antiviral activity at 1.56 µl/ml, and was still active at 0.1µl/ml dilution. Carvacrol itself showed antiviral activity intermediate between the ORE oils and EP. Table 2 shows their relative IC_{50} values (concentrations giving 50% inactivation of virus).

Table. 2: Antiviral IC_{50} Values

Test material	IC_{50}
Echinacea purpurea (EP)	0.05
ORE-1, *O.minutiflorum*	5.93
ORE-2, *O. minutiflorum*	24.17
ORE-3, *O. vulgare*	11.9
Carvacrol	2.6

IC_{50} = dilution giving 50% inhibition of virus plaques

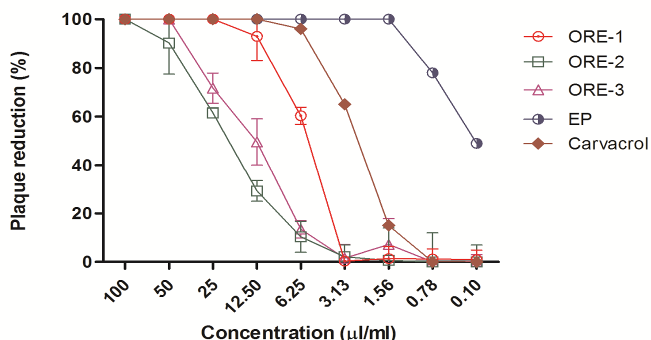

Fig. 1 : Antiviral Activities of serial dilutions of Oregano Oils (ORE 1-3), carvacrol and EP. Each sample, in triplicate, was serially diluted 2x and incubated with a standard amount of H1N1 virus (1000 pfu per reaction). Remaining infectious viruses were measured by plaque assay on MDCK cells.

Antiviral Effects at High Virus doses

In subsequent experiments the carrier-free oils ORE-4 and ORE-5, and pure olive and canola oils, were acquired. Initially the antiviral tests with ORE-4 and -5 were complicated by their cytotoxicity at high concentrations. These oils were therefore tested by means of a modified protocol in which larger amounts of virus (10^5 pfu) were incubated with several concentrations of oil and the reactions then diluted 50-100-fold to obtain measurable numbers of pfu. (viral plaques, table 3). In these assays ORE-4 and -5 were found to be antiviral, approximately the same potency as carvacrol, though always much less than EP (data not shown). To our surprise pure olive oil also showed significant antiviral activity, indicating that the so-called “carrier oil” was by no means inert. However olive oil did not appear to affect significantly the antiviral activity of pure oregano oil, as illustrated by the results of mixing experiments (Table 3). In contrast Canola oil showed no antiviral activity even at high concentration.

Table 3. : Antiviral activities of oils and mixtures (10^5 pfu per reaction)

	Concentration (µl/ml)	Inhibition (%)
EP	10 5	100 100
ORE-4	10 5	100 0
ORE-4 + Olive oil mix	100 10 5	100 98±5 0
ORE-5	10 5	100 0
Olive oil	100 10	100 0
Carvacrol	10	100
Canola oil	100	0

Cytotoxicity of oils

Since the recommended Oregano applications involve exposure of oral and pulmonary epithelial cells to the oils, we decided to evaluate cytotoxicity in human lung epithelial cells (A549), following short (10 min) or long (24h) exposures, by means of the XTT assay technique for cell viability. Results for 10 min exposures to Oregano oils are shown in Figures 2 and 3. ORE-1-3 showed some toxicity at the high concentrations but was non-toxic at concentrations less than 25 µl/ml. In contrast ORE-4 and 5 were toxic even at 0.16 µl/ml. Fig. 4 shows the corresponding results for EP and olive oil, which were essentially non-toxic, and carvacrol, which was substantially cytotoxic. The 24 hour exposures showed greater degrees of cytotoxicity, except for olive oil, which remained non-cytotoxic. These data are summarized as TC_{50}’s in Table 4 for both 10 min and 24 hour exposures.

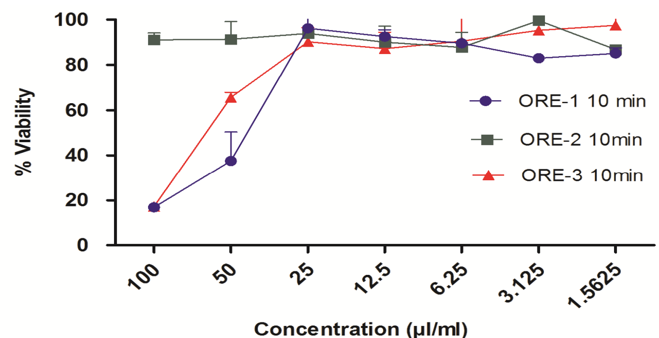

Figure 2. : Cytotoxicity of Oregano oils 1-3; short exposures
Monolayers of human lung epithelial cells (A549) in 96-well trays were incubated with the indicated concentration of oil in medium, for 10 min followed by normal medium for 24 h. Controls were incubated in normal medium. All cultures were then assayed for cell viability by the XTT method described in Materials and Methods.

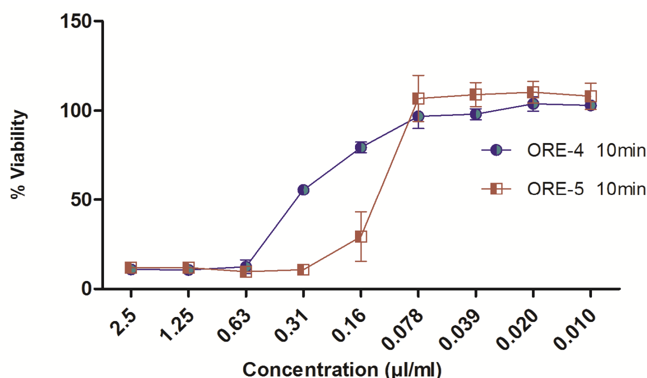

Fig. 3: Cytotoxicity of Oregano oils without carriers (ORE 4-5); short exposures Monolayers of human lung epithelial cells (A549) in 96-well trays were incubated with the indicated concentration of oil in medium, for 10 min followed by normal medium for 24 h. Controls were incubated in normal medium. All cultures were then assayed for cell viability by the XTT method described in Materials and Methods.

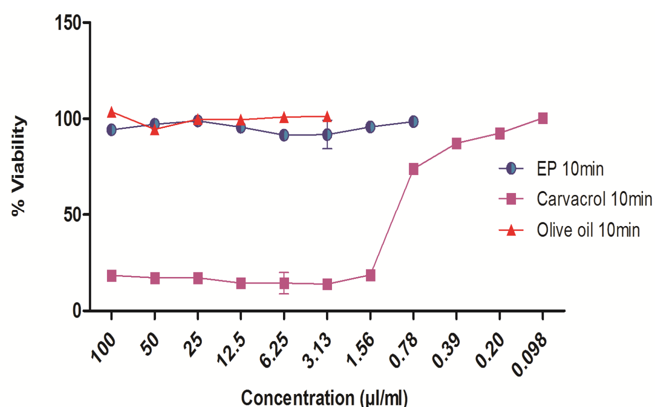

Fig. 4: Cytotoxicity of Carvacrol and Olive oil (compared with EP) Monolayers of human lung epithelial cells (A549) in 96-well trays were incubated with the indicated concentrations of carvacrol, olive oil, or reference Echinacea (EP) in medium, for 10 min followed by normal medium for 24 h. Controls were incubated in normal medium. All cultures were then assayed for cell viability by the XTT method described in Materials and Methods.

Table. 4: TC_{50} values of oils in human lung epithelial cells

	TC_{50} (µl/ml) [1]	
Samples	**Viability (24h exposure)**	**Viability (10min exposure)**
ORE-1	2.63±0.05	49.06±0.195
ORE-2	1.25±0.03	>100
ORE-3	0.625±0.006	49.94±0.115
ORE-4	0.09±0.002	0.039±0.002
ORE-5	0.08±0.0025	0.101±0.007
EP	25±3.23	>100
carvacrol	0.000019	0.4±0.049
Olive oil	>100	>100

DISCUSSION

"Oil of oregano" is a popular natural health product, but little research seems to have been conducted on its antiviral activity, although antibacterial activities have been well documented (Burt 2004, Sokovic *et al*., 2010, de Souza *et al*., 2010). However some other essential oils have demonstrated antiviral activities, such as Tea Tree oil (Carson *et al*., 2006), Salvia (Alim *et al*., 2009), and Eucalyptus oils (Cermelli *et al*., 2008, Sadlon and Lange, 2010). Nevertheless the popular health literature contains abundant claims to the efficacy of Oil of Oregano (not always *O. vulgare*) in counteracting colds and 'flu, especially pandemic H1N1, in spite of the dearth of scientific evidence, and the inability of Sokmen et al (2004) to find anti-influenza virus activity in their extracts derived from *O. vulgare*.

The studies reported here have shown that five commercial Oregano oils, including two preparations without carrier, do indeed possess significant anti-influenza virus activity, although much less potent than the reference *Echinacea* preparation (EP). EP was used as a known potent anti-viral extract, against which we can evaluate other prospective phytomedicines for antimicrobial and immune modulatory activities (Pleschka *et al*., 2009, Vimalanathan *et al*., 2009). However the presence of carrier olive oil in most of the commercial Oregano preparations raised the issue of its possible role in the antiviral activity, either as a negative or positive influence. These tests showed that olive oil by itself contributed significant activity against H1N1, but would not be expected to affect the overall antiviral performance of the final product. Carvacrol, the main constituent of oregano oil, was by itself very active, but was also very cytotoxic. These results support the rationale for adding olive oil to Oregano oils with high carvacrol content.

Antiviral activities against human influenza viruses are usually carried out in MDCK cells, an established line of canine kidney epithelial cells, which support the growth of the virus in the presence of trypsin (required by most human influenza virus stains, WHO,2011). This was the cell line used in our tests. Theoretically one could carry out cytotoxicity tests in this same cell line (in the absence of trypsin), and in fact the antiviral assays themselves did indicate cytotoxic effects of all the Oregano oils at the higher concentrations, similar to those used in consumer applications. However, since Oregano oils are normally recommended for oral applications to control colds and 'flu, we decided to evaluate cytotoxicity in a more relevant cell line, the A549 human lung epithelial line. A short exposure of 10 min was chosen to represent a typical oral application to the oral or nasal mucosa, and the longer exposure of 24 h represented a standard cytotoxicity test. Carvacrol alone displayed significant cytotoxicity, even in the short exposure protocol, as did the carrier-free ORE-4 and 5, whereas the other oils were less toxic, especially during the short exposure. Thus other components of the oils must have some protective property that counteracts the substantial toxic effect of carvacrol. In contrast olive oil by itself, and the *Echinacea* preparation, showed relatively little effect on the cells.

The apparent variation in antiviral and cytotoxic activities between the five Oregano oils could be a reflection of slight differences in source materials or the final composition of the oils, since antiviral activity could depend on more than simply carvacrol content. In other recommended uses of Oregano oils, such as skin application for the control of fungal and bacterial infections, and as drinks diluted in water or juice, the demonstrated cytotoxic effects might be less important.

CONCLUSION

Thus there is a basis for the claim of anti-influenza virus activity of some Oregano oils, although this activity was not

comparable to a potent standard *Echinacea purpurea* preparation, and furthermore their potential cytotoxicity to lung epithelial cells is a limiting factor in their oral applications. Carvacrol by itself was also antiviral, but was also extremely cytotoxic. Consequently the difference between minimal cytotoxicity and significant antiviral activity was relatively small for the Oregano oils, in comparison with a much larger difference for *Echinacea purpurea*.

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