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PHYTOPHARMACEUTICAL PREPARATION OF ESSENTIAL OIL (*PPEO*) PROMOTES AN ANTI-INFLAMMATORY RESPONSE INDUCED BY INFLUENZA A/H1N1 PDM09 VIRUS INFECTION *IN VIVO*

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Abstract: Influenza is an acute self-limited respiratory illness, characterized by inflammatory response, pulmonary infiltrates, vasodilator shock and high mortality. Treatment is focus to the use of a limited set of anti-viral drugs. However, this therapy is expensive and frequently cases of viral drug resistance have been documented. In the present study using clinical scale and histopathological and immunofluorescence analyses, we evaluated the prophylactic and therapeutic effects of an Phytopharmaceutical Preparation of Essential Oil (*PPEO*), based on cinnamon, guava, propolis, and menthol on influenza A murine model. Both, prophylactic and therapeutic treatments showed moderate clinical signs, as labored breathing, tremors, lethargy. Histopathologically, we observed that the pulmonary architecture is preserved in 80% and 90 % of the alveoli, respectively with no evidence of pulmonary parenchymal consolidation, displaying a very limited inflammatory process. We found only a slight interstitial pneumonitis with few lymphocytic infiltrate in both groups in constrast to infected animals without treatment. Immunofluorescence analysis showed that decrease in the inflammatory process correlated with decrease of Nuclear Factor-kB (NF-kB) translocation to the nucleus. The formulation has a local anti-inflammatory effect, suggesting its potential as coadyuvant treatment for influenza.
Keywords: Influenza A, inflammation, cytokines, NF-kB, *in vivo* model.

INTRODUCTION

Influenza is an acute respiratory illness that solves in almost five days; however, infection can cause severe systemic inflammatory response, pulmonary infiltrates, vasodilator shock and high mortality (Julkunen *et al.*, 2001; Who, 2010; Buttignol *et al.*, 2017; Guo and Thomas, 2017). The intense inflammatory

responses, occurs mainly in young and apparently healthy people, suggesting that in addition to the load and pathogenicity of the virus (Hui *et al.*, 2018), other host genetic factors (Choudhary *et al.*, 2018), producing a hyper-immune response could generate a pro-inflammatory state with presence to cytokines as interferons (IFN´s), IL-1β, IL-18, TNF-α, IL-6 and "alarmina" IL-33 (Kwang Seok Kim, Hyemin Jung, In Kyung Shin, Bo-Ra Choi, 2015; Guo and Thomas, 2017; Teijaro, 2017). To reduce morbidity and mortality, a variety of public health interventions have been implemented (Cowling, B.J., Chan, K.H., FAng, V.J., Cheng, 2009; Who, 2010; Hui *et al.*, 2018). Vaccines are the most widely used intervention for influenza infection prophylaxis (Collin and de Radiguès, 2009), but their effectiveness depends on the type of influenza virus involved in each seasonal epidemic (avian influenza A/H5N1, A/H7N9 and seasonal A/H1N1 pdm09 virus infections), even cause hypercitokinemia (Teijaro, 2017; Hui *et al.*, 2018). On the other hand, a limited set of drugs for symptomatic patients are available; however, there are many restricted factors such as neurotoxicity, gastrointestinal side effects and drug resistance. The emerge of pandemic 2009 A/H1N1 Influenza Virus (pdm2009 A/H1N1) and others viral strains have prompted new global efforts to find other ways to reduce the health impact of influenza infection in animal models including anti-inflammatory strategies as sphingosine 1 phosphate modulators, COS inhibitors, PPAR (peroxisome proliferator-activaed receptor) agonists (Siqueira *et al.*, 2016; Teijaro, 2017; Hui *et al.*, 2018), homeopathic treatments as thymulin, influenza biotherapies (Siqueira *et al.*, 2016), and natural compounds from medicinal plants (Xu and Liu, 2017; Mehrbod *et al.*, 2018). Mehorbod *et al* in 2018, showed that the compound quercetin-3-O-α-L-rhamnopyranoside (Q3R) from *Rapanea melanophloeos* has antiviral and immunomodulatory activity against influenza A virus by its decreased NP and M2 gene expression in *in vitro* study (Mehrbod *et al.*, 2018). Finally, Ximing y Liu elucidated the mechanism of cucurmin in influenza A virus infection through inhibiting the NF-κB signaling pathway (Xu and Liu, 2017).

Here, to search for other phytopharmacological components anti-influenza, we report the prophylactic and therapeutic effects of Phytopharmaceutical preparation of essential oil (*PPEO)* based on cinnamon, guava, propolis and menthol in *in vivo* influenza A/H1N1 pdm09 murine model.

MATERIAL AND METHODS

MICE

Female Balb/C mice were obtained from CINVESTAV-IPN México. All animals were 6-8 weeks old with average weights between 18 to 20 g were used for viral passage and antiviral activity of PEO. The animals had free food and water access and subject 12 h light/dark cycles, receiving humane treatment based on the criteria of the International Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources Committee, 2011) also according to the Mexican Guidelines for the Production, Care and Use of Laboratory Animals (NOM-062-ZOO-1999).

INFECTION AND VIRUS PASSAGES

Human influenza A/H1N1 pdm09 virus strain was kindly provided by the laboratory of Ph. D Carlos Cabello (INER, SSA México). All virus used for the experiments came from a single viral preparation batch prepared in Madin-Darby Canine Cells (MDCK). Mice were anesthetized with ketamine and xilazine (50 mg/kg, 5 mg/kg, respectively) in 100 µL of distilled water and the mice were infected intranasally with 66 µl of virus with a $1x10^5$

Spearman & Kärber title with a 100% lethal dose (LD_{100}) (8). Animals were weighted and observed daily for 4 days and sacrificed to obtain the lungs. The lungs were homogenized with 500 µL of MEM medium and centrifuged at 10,000 × g at 4°C for 15 min. Eighty µL of lung homogenate were used for second and third infection passages to obtain influenza A/H1N1 pdm09 adapted to mouse (Menéndez, Peña and Hernández, 2005).

PPEO BY HIDRODISTILLATION.

PPEO was obtained by hydrodistillation as reported previously (Calvo-Gómez, Morales-López and López, 2004), briefly, 14 kg of formulation (2 kg of propolis, camphor and cinnamon respectively of the PROQAVIF S.A. de C.V and 8 kg of guavas) were prepared with 5 liters of water for 20 min in an industrial blender (Santos model Miss Blend, Mexico). Then, formulation was introduced into a distillation apparatus and heated for 12 h in a double boiler until the temperature reached 58 °C. The pressure was then slowly reduced from 250 to 150 mm Hg, so the vapors were condensed and the oils were separated, the procedures were repeated four times. *PPEO* were collected and divided in 1 ml Pyrex glass flasks (catalog number 5640) and storing at −20 °C.

PPEO CHARACTERIZATION BY GAS CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY (GC-MS)

One µl of PPEO/dichloromethane dilution (ratio of 2:48) was injected and analyzed in a HP 5890 Series II gas chromatograph directly coupled to an HP 5890 mass-selective detector (Hewlett-Packard, Palo Alto, CA, USA). A HP-FFAP (30 m×0,25mm i.d., film thickness 0,25 m; Hewlett-Packard) capillary column was used. A split injector was used at a ratio of 1:2; the injector and detector temperatures were 200 °C and 250 °C, respectively. Experimental conditions were as follows: column temperature was held at 60 °C for 3 min and then increased at 0,083 $°Cs^{-1}$ to 250 °C; helium was used as carrier gas at a linear flow of 33 l s^{-1}. Mass spectra was obtained at 70 eV and the ion source was at 250 °C. Assays were performed three times. The identification of the analyzed compounds was accomplished by comparing their mass spectra with those of authentic compounds available from computerized spectral database (NIST/EPA/NIH 75K), or from published literature. The percentage content was calculated by peak area normalization(Calvo-Gómez, Morales-López and López, 2004).

PPEO ADMINISTRATION IN A MOUSE MODEL OF INFLUENZA A AND CLINICAL ANALYSIS

For therapeutic administration 6 animals were anesthetized with ketamine and xilazine (50 mg/kg, 5 mg/kg respectively) in 100 µL of distilled water and infected by nostril instillation with 80 µL of virus from third pass. Twenty-four hours after infection, animals were instilled with: 16,3 x 10^{-3} ml/g mouse/day of *PPEO* diluted in 5% Tween 80 for four days. For prophylactic administration, 6 animals were treated with 16,3 x 10^{-3} ml/g mouse/day of PPEO diluted in 5% Tween 80, 7 days before infection. As controls, infected and non-infected animals were instilled with 80 µL of 5% Tween 80 saline solution. Animals were observed weight daily for 7 days, to register the clinical signs by modified arbitrary scale of Menendez and coworkers in 2005 (Menéndez, Peña and Hernández, 2005). After, animals were sacrificed 7 days post-infection, and lung tissue was obtained from each animal, fixed in 10% formaldehyde or 4% paraformaldehyde for histopathological and immunofluorescence analysis, respectively.

HISTOPHATOLOGICAL ANALYSIS

To visualize different components of the lung under a microscope, the sections are dyed with Hematoxylin and eosin (H&E) (Sigma Aldrich, St Louis, MO, USA). Lungs was fixed in formaldehyde/buffer 10%, dehydrated with ethanol washes (70 %, 90 % and 100%), cleared with xylene and included in paraffin at 60 °C. Sections of 3 µm thickness were made in rotary microtome (American Optical 820) and mounted on slides. Then, histologic sections were immersed in xylene to remove excess paraffin. Tissue sections were rehydrated by passing through a decreasing concentration gradient of alcohol and water baths (100%, 90%, 80%, and 70%). Subsequently, the tissue sections were immersed in hematoxylin for 10 minutes and rinsed in tap water until the sections exhibited a blue coloration. The tissue sections were immersed in 1% alcoholic acid (1% H Cl in 70% alcohol) for 5 minutes. Then, the histological sections were washed in running water, placed into the eosin for 30 seconds and treated with another series of alcohol baths, in increasing order (70%, 95%, and 100%) (Acosta *et al.*, 2016).

IMMUNOFLUORESCENCE ANALYSIS OF NF-κB.

Tissue sections fixed in 4% paraformaldehyde/PBS, pH 7.4 were soaked in 10% sucrose / PBS overnight 5 µm lung thickness were made in a Leica cryostat (CM30505), mounted on gelatinized slides, and stored frozen at -20° C. The cryosections were rehydrated with cold PBS pH 7,4 for 10 min. Cytoplasmic RNA was remove with 5 mM ammonium chloride for 10 minutes and 1 M HCl for 2 h at room temperature. Samples were permeabilized with Triton X-100 PBS 0.2% and blocked with BSA-free 0.2% IgG for 20 min to remove nonspecific labeling. Inmunodetection was done using 50 µL of anti NF-κB primary antibody (NFκB p100/p52; ABCAM) at 1:50 dilution in PBS-Tween 80, for 2 h and this was followed by incubation with fluorescein isothiocyanate (FITC)-rabbit anti-goat immunoglobulin (Ig) G antibody (1:60; Jackson Immuno Research, West Grove, PA, USA) for 1 h at room temperature. Counter staining was performed with propidium iodide (1:1000; Vector Laboratories, Burlingame, CA, USA) for 5 minutes and mounted with Vectashield and sections were sealed with nail polish, keeping cuts at 4°C. Samples were analyzed using laser scanning confocal microscope (LSM 710, Carl Zeiss, Germany, Center for Nanoscience and Nanotechnology, IPN). The image acquisition was made under the following conditions: Best Signal Mode, Scan Mode Plane (2D), Plan-Apochromat objective 63x/1,40 Oil DIC M27, using a pseudo colour display green (FITC) and red (propidium iodide, PI). Fluorochromes in double labelled samples were excited at wavelengths of 488 nm (for FITC) and 554 nm (for PI).

We use a negative control of fluorescence without NF-κB antibody (Acosta *et al.*, 2016).

RESULTS

CHEMICAL COMPOSITION OF THE *PPEO*

Taking in consideration the empirical clinical effect of poli-herbs formulation, constituted by grinded licorice, cinnamon, guava, propolis, and menthol, in diminishing the symptoms of flu, here we produced an *PPEO* of the mixture by hydrodistillation. Prior to antiviral studies, the chemical composition of oil was investigated by GC-MS. The results are showed in Table I. Nineteen compounds were identified, making up 84 % of the total composition. Major components were menthol (31,44%), phenyl ethyl acetate (5,72%), cinnamaldehyde (5,11%), limonene (5,04%) and linalool (5,02%).

	Compound	RT	Relative Abundance%
1	Limonene	5,62	5,04
2	prenyl acetate	6,86	2,57
3	Cymene	7,20	1,89
4	(E)-2-methylbut-2-en-1-ol	8,78	1,04
5	Linalool	14,6	5,02
6	α-guaiene	15,40	3,33
7	Mentol	17,1	31,44
8	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	17,52	3,60
9	β-himachalene	17,82	3,17
10	Eremophilene	18,42	2,51
11	PI*	18,63	2,64
12	Benzylacetate	19,24	2,41
13	δ-cadinene	19,83	2,09
14	PI*	20,00	1,01
15	Curcumene	20,55	2,40
16	Phenyethylacetate	22,53	5,79
17	PI*	24,58	1,60
18	Cinnamaldehyde	28,11	5,11
19	PI*	29,89	1,50
	Total		84,16 %

* PI, Partially identified

Table I. Chemical composition of PPEO.

INDUCTION OF SEVERE RESPIRATORY ILLNESS BY INFLUENZA A/H1N1 PDM09 VIRUS INFECTION *IN VIVO*

To study the effect of *PPEO* administration on influenza A, first, we produced pdm2009 A/H1N1 influenza virus adapted to mouse able to produce severe respiratory illness with an intense inflammatory response, animals had a gradual deterioration in health status.

We showed that non-infected animals had not signs of disease with a normal lung architecture, thin interalveolar walls, alveoli preserved with thin walls in bronchi and vessels (Figure 1A). Interestingly, the results showed that human influenza A/H1N1 pdm09 virus was adapted to mice by doing three serial pass infections. First pass presented light lymphocytic infiltrate, septal congestion, intra-bronchial and peri-bronchial hemorrhage (Figure 1, B); animals from second pass decreased the body weight (17 % in average) and displayed after 72 h of infection, bristling, mainly in the neck. Histologically, lung parenchyma showed interstitial alterations characterized by

thickening of the alveolar walls, confluence of the air spaces and an important lymphocytic inflammatory infiltrate (Figure 1, C). While animals from third pass decreased body mass (30 %) at 72 h post-infection, showing ruffling, lethargy, an important difficulty in breathing and cyanosis in tail and ears. In figure 1D, the histopathological changes of the third pass presented an acute interstitial pneumonitis, light bleeding, deformation of cells, peribronchial and septal congestion and increased lymphocytic infiltrates.

PPEO-TREATED MICE DIMINISHED THE SEVERE RESPIRATORY MANIFESTATIONS BY INFLUENZA A/H1N1 PDM09 VIRUS INFECTION *IN VIVO*

PPEO were administered prophylactically some improvement were observed clinical signs. Animals treated with *PPEO* were sacrificed to evaluate structural architecture of lungs. Pulmonary tissue from non-infected mice (Figure 2, A) showed architecture of normal tissue, constitute of alveoli with thin walls formed by pneumocytes type I and II; no evidence of inflammation, nor alveolar damage were found. In counterpart, as we showed before, influenza infected mice presented the severe signs of influenza A. In the group (AH1N1+ V_{PPEO}), animals presented forced breathing, tremor and lethargy, decreased mobility, and cyanosis in tail and ears and intense inflammatory response. The structure forming the alveoli was replaced by inflammatory tissue in more than 80% of its area, becoming confluent alveoli with thickened walls (Figure 2, B), as reported Menendez y colaborators in 2005. In contrast, animals administered prophylactically (PPEO+AH1N1) and therapeutically (AH1N1+ PPEO) by intranasal *PPEO*, we observed the 100% of the animals during sixth and seventh day post infection presented moderate clinical signs, as labored breathing, tremors, lethargy after both treatment. Histopathologically, we observed that the pulmonary architecture is preserved in 80% and 90 % of the alveoli preserved respectively, the alveolar sacs have a characteristic elongated morphology are seen, we found only a slight interstitial pneumonitis with slight lymphocytic infiltrate in both groups (Figure 2 C-D).

PPEO DECREASE OF INFLAMMATORY PROCESS BECAUSE NF-κB IN INFLUENZA A/H1N1 PDM09 VIRUS INFECTION *IN VIVO*

To explore the putative mechanism involved in the anti-inflammatory effects observed during the prophylactic administration of PPEO, we immunodetected NF-κB in lung tissues from the different groups of treatment. Results showed a basal expression of NF-κB in normal lung tissue, distributed in less than 5% of the cytoplasm of epithelial cells without nuclear staining (Figure 3, A). On the other hand, NF-κB expression was very intense in lungs from influenza infected animals without any treatment. It was observed mainly in the cytoplasm from 90-100% of the epithelial cells (Figure 3, B). Figure 3, C shows the histological section of lung from an infected animal therapeutically treated with PPEO (AH1N1+PPEO). In this group, NF-κB was observed in 15-20% of the histological section. Tridimensional analysis of nuclei showed that some nuclei staining was co-localizing with NF-κB fluorescence. Interestingly, expression of NF-κB in lungs from infected animals, administrated prophylactically with PPEO 7 days before infection (PPEO+AH1N1), showed that fluorescence was dramatically diminished displaying a similar pattern in intensity and location to lung epithelium from non-infected animals (V_{AH1N1}+V_{PPEO}) (Figure 3D).

DISCUSSION

The severity of illness during influenza infection is a function of direct virus-induced pathology as well as host innate and adaptive immune response (Guo and Thomas, 2017). The developments of novel therapeutic alternatives that help counteract clinical manifestations and immunopathology, i. e. complementary therapies for the prevent severe cases by influenza virus infection (Fukuyama and Kawaoka, 2011; Yuan, 2013). Here, to evaluate immunomodulators properties of the *PPEO* against clinical manifestations and lung pathology produced by pdm2009 A/H1N1 Influenza Virus in a mouse model. Laboratory research has found that many essential oils diminish viral infectivity. For example, different essential oils attenuate influenza virus infection *in vitro* (Wu S, et al. 2010).

Here, to characterize the effect of PPEO, we used influenza A/H1N1 pdm09 virus infection *in vivo* mouse model. The infection of mice using human pdm2009 A/H1N1 *Influenza Virus* directly, produced no clinical symptoms (first pass); second pass increased the clinical manifestations; while virus from third pass displayed and important manifestations (Menéndez AB, et al. 2005). Important deterioration of animal health status displaying severe pathological manifestation in lungs, was very similar to that found in humans (Menéndez AB, et al. 2005). Some reports have showed that the histopathology of primary pneumonia caused by influenza in humans is characterized by an intense vascular congestion, dilation and thickening of the alveolar septum. Within the alveoli also is described the presence of neutrophils, alveolar macrophages and erythrocytes scarce, appearing wall of the alveoli lined by hyaline membranes and thick eosinophilic (Carrada T. 2011) and in patients with diffuse alveolar damage had higher expression of CD8+ and CD4+ T cells, and CD83+ dendritic cells, granzyme A+ and natural killer and cell density in the lung parenchyma, indicating a cytotoxic inflammatory phenotype (Buttignol *et al.*, 2017). In our study, we observed histopathological similarities, showing a higher degree of damage, suggesting that the virus adapted to mice is highly virulent. After therapeutic or prophylactic administration of *PPEO*, the important pneumonic process observed in third pass infection mice was decreased. Interestingly, the effect was enhanced in those animals administered prophylactically with slight lymphocytic infiltrate and conservative pulmonary morphology. Particularly, we found an important anti-inflammatory effect of *PPEO*. Several reports have shown that during host viral replication, different signaling pathways are involved; one of them is the NF-kB pathway whose expression has been demonstrated in the early stages of infection(Go *et al.*, 2011), where the cells produce IFN-α/β and other pro-inflammatory cytokines activated by NF-kB transcription factor (Julkunen *et al.*, 2001; Guo and Thomas, 2017), but after of chronic viral infection, there are exacerbate immune response by patient and it entails hipercitocinemia and exacerbate inflammation (Guo and Thomas, 2017; Hui *et al.*, 2018). It has been observed that overexpression of NF-kB correlates with an exacerbated inflammation in the lung (Julkunen *et al.*, 2001). In our model, lung histopathological analyses of infected organisms showed an important inflammatory process in concordance with a highly NF-kB expression. In counterpart, *PPEO* treatment, particularly, prophylactic administration, showed a limited pathological manifestation with an important reduction of inflammation, correlating it, with a dramatically reduction of NF-kB expression, suggesting that activation of NF-kB by viral infection may be being limited

by some of the essential oil components. GM-MS of the PPEO showed that majority compounds were menthol (31.44 % relative abundance), cinnamaldehyde (5.11 % relative abundance), linalool (5.02 %), acetic acid, 2 phenylethyl-ester (5.72 %), caryophyllene (3.84 %) and limonene (5.02 %). *In vitro* studies of some of these compounds have demonstrated anti-inflammatory effects (Juergens UR, et al. 1998, Chao LK, *et al*. 2008, Peana AT, et al. 2002, Yoon WJ, et al. 2010, Yoon WJ, et al. 2010, Hiroto R, et al. 2010). Juegens et al. (1998) using lipopolysaccharide (LPS) stimulated monocytes from human volunteers, observed that menthol significantly suppressed the production of PGE and IL-1β in these cells. Similar results were observed using cinnamaldehyde (CNA) on murine macrophages stimulated with LPS or lipoteichoic acid (LTA), where in the compound, administered at low doses (μM), inhibited the secretion of IL-1β and TNF-α (Juergens UR, et al. 1998). Results from these studies propose the anti-inflammatory activity of the CNA through reduction of oxidative stress, observing the inhibition of NIK/IKK, ERK and p38 MAPK pathways. Linalool and its corresponding acetate form also have an important role in the anti-inflammatory activity of different essential oils, using it as treatment in a carrageenin induced edema model (Peana AT, et al. 2002). Caryophyllene administered at a dose of 12.5 and 25 mg/kg showed a significant analgesia accompanied by an anti-inflammatory activity (Peana AT, et al. 2002). On the other hand, limonene has an inhibitory effect of nitric oxide (NO) and LPS-induced production of prostaglandin E (2) in macrophages, as well as in the secretion of cytokines such as TNFα, IL-1β and IL-6 (Yoon WJ, et al. 2010). Interestingly, investigation on human cells eosinophilic leukemia HL-60, showed that at low concentrations (7,34 mmol/l), limonene inhibited the production of ROS while at 14,68 mmol/l, decreased the production of MCP-1 via NF-kB activation (Hiroto R, et al. 2010). These evidences of anti-inflammatory activities of compounds found in *PPEO*, strongly support the observed anti-inflammatory effects in our influenza model, diminishing the clinical and histopathological manifestations of disease, allowing a better recovery in *PPEO* treated animals. With the systematic research and understanding of the antiviral mechanisms of remedies, more traditional medicines will be utilized for clinical pharmaceutical purposes and novel drug discoveries will be done. The lack of toxicity and potent specific viral inhibitory activity suggest that *PPEO* may be helpful as a putative antiviral drug for the control and treatment of influenza infections. Since the *PPEO* was obtained, is currently used as supplement orally administrated, once the pharmacokinetics will be determined, it will may provide therapeutic benefit during some states of influenza infection.

CONCLUSION

In summary, our results indicate that *PPEO* modifies influenza-related clinical course and inflammatory response decreasing pulmonary infiltrate and decreasing the NF-κB expression levels.

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REFERENCES

Acosta, P. *et al.* (2016) 'Anti-inflammatory effect of dialysable leucocyte extract in a rat model of osteoarthritis : histopathological and molecular characterization Anti-in fl ammatory effect of dialysable leucocyte extract in a rat model of osteoarthritis : histopathological an', 9742(April). doi: 10.3109/03009742.2016.1153140.

Buttignol, M. *et al.* (2017) 'Airway and parenchyma immune cells in influenza A(H1N1)pdm09 viral and non-viral diffuse alveolar damage', *Respiratory Research*. Respiratory Research, 18(1), pp. 1–10. doi: 10.1186/s12931-017-0630-x.

Calvo-Gómez, O., Morales-López, J. and López, M. G. (2004) 'Solid-phase microextraction-gas chromatographic-mass spectrometric analysis of garlic oil obtained by hydrodistillation', *Journal of Chromatography A*, 1036(1), pp. 91–93. doi: 10.1016/j.chroma.2004.02.072.

Carrada T. 2011. Influenza humana: avances recientes en la patogenia e histopatología. Descripción del brote pandémico en México 2009-2010. *Rev Latinoamer Patol Clin.* 58: 60-101.

Chao LK, Hua KF, Hsu HY, Cheng SS, Lin IF, Chen CJ *et al.* 2008. Cinnamaldehyde inhibits pro-inflammatory cytokines secretion from monocytes/macrophages through suppression of intracellular signaling. *Food Chem Toxicol.* 46: 220-31. doi: http://dx.doi.org/10.1016/j.fct.2007.07.016

Chavan MJ, Wakte PS, Shinde DB. 2010. Analgesic and anti-inflammatory activity of caryophyllene oxide from Annona squamosal L. bark. *Phytomedicine.* 17:149-51. doi: http://dx.doi.org/10.1016/j.phymed.2009.05.016.

Choudhary, M. L. *et al.* (2018) 'Association of single nucleotide polymorphisms in TNFA and IL10 genes with disease severity in influenza A/H1N1pdm09 virus infections: A study from Western India', *Viral Immunology*, 31(10), pp. 683–688. doi: 10.1089/vim.2018.0120.

Collin, N. and de Radiguès, X. (2009) 'Vaccine production capacity for seasonal and pandemic (H1N1) 2009 influenza', *Vaccine*, 27(38), pp. 5184–5186. doi: 10.1016/j.vaccine.2009.06.034.

Cowling, B.J., Chan, K.H., FAng, V.J., Cheng, C. K. Y. (2009) 'Annals of Internal Medicine Article Facemasks and Hand Hygiene to Prevent Influenza Transmission in Households', *Annals of Internal Medicine*, (151), pp. 437–446.

Fukuyama, S. and Kawaoka, Y. (2011) 'The pathogenesis of influenza virus infections: the contributions of virus and host factors.', *Current opinion in immunology*, 23, pp. 481–486. doi: 10.1016/j.coi.2011.07.016.

Go, Y.-M. *et al.* (2011) 'Increased inflammatory signaling and lethality of influenza H1N1 by nuclear thioredoxin-1.', *PloS one*, 6(4), p. e18918. doi: 10.1371/journal.pone.0018918.

Guo, X. zhi J. and Thomas, P. G. (2017) 'New fronts emerge in the influenza cytokine storm', *Seminars in Immunopathology*. Seminars in Immunopathology, 39(5), pp. 541–550. doi: 10.1007/s00281-017-0636-y.

Hiroto R, Roger NN, Nakamura H, Song HS, Sawamura M, Suganuma N. 2010. Anti-inflammatory effects of limonene from yuzu (Citrus junus Tanaka) essential oil on eosinophils. *J Food Sci.* 75: 87-92. doi: http://dx.doi.org/10.1111/j.1750-3841.2010.01541.x.

Hui, D. S. *et al.* (2018) 'The role of adjuvant immunomodulatory agents for treatment of severe influenza', *Antiviral Research*. Elsevier, 150(November 2017), pp. 202–216. doi: 10.1016/j.antiviral.2018.01.002.

Institute of Laboratory Animal Resources Committee (2011) *Guide for the Care and Use of Laboratory Animals*, *Guide for the Care and Use of Laboratory Animals*. doi: 10.2307/1525495.

Juergens UR, Stöber M, Vetter H. 1998. The anti-inflammatory activity of L-menthol compared to mint oil in human monocytes in vitro: a novel perspective for its therapeutic use in inflammatory diseases. *Eur J Med Res.* 3: 539-45.

Julkunen, I. *et al.* (2001) 'Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression.', *Cytokine & growth factor reviews*, 12(2–3), pp. 171–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11325600.

Kwang Seok Kim, Hyemin Jung, In Kyung Shin, Bo-Ra Choi, and D. H. K. (2015) 'Associated With Antiviral Therapy', *Journal of Medical Virology*, 87, pp. 1104–1112. doi: 10.1002/jmv.24138.

Mehrbod, P. *et al.* (2018) 'Immunomodulatory properties of quercetin-3-O-α-L-rhamnopyranoside from Rapanea melanophloeos against influenza a virus', *BMC Complementary and Alternative Medicine*. BMC Complementary and Alternative Medicine, 18(1), pp. 1–10. doi: 10.1186/s12906-018-2246-1.

Menéndez, A. B., Peña, B. D. R. and Hernández, E. A. (2005) 'Aspectos clínicos y virológicos útiles en la implementación de un modelo de Influenza A en líneas de ratones Balb/C', *Acta Farmaceutica Bonaerense*, 24(3), pp. 395–401.

Peana AT, D'Aquila PS, Panin F, Serra G, Pippia P, Moretti MD. 2002. Anti-inflammatory activity of linalool and linalyl acetate constituenst of essential oils. *Phytomedicine*. 9: 721-26. doi: http://dx.doi.org/10.1078/094471102321621322

Siqueira, C. M. *et al.* (2016) 'Homeopathic treatments modify inflammation but not behavioral response to influenza antigen challenge in BALB/c mice', *Homeopathy*, 105(3), pp. 257–264. doi: 10.1016/j.homp.2016.04.002.

Teijaro, J. R. (2017) 'Cytokine storms in infectious diseases', *Seminars in Immunopathology*. Seminars in Immunopathology, 39(5), pp. 501–503. doi: 10.1007/s00281-017-0640-2.

Who (2010) 'WHO Guidelines for Pharmacological Management of Pandemic Influenza A(H1N1) 2009 and other Influenza Viruses', *WHO Guidelines for Pharmacological Management of Pandemic Influenza A(H1N1) 2009 and other Influenza Viruses*, (February), pp. 1–32. Available at: http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:WHO,2010#7.

Wu S, Patel KB, Booth LJ, Metcalf JP, Lin HK, Wu W. 2010. Protective essential oil attenuates influenza virus infection: an in vitro study in MDCK cells. *BMC Complement Altern Med.* **10:** 69. doi: http://dx.doi.org/10.1186/1472-6882-10-69.

Xu, Y. and Liu, L. (2017) 'Curcumin alleviates macrophage activation and lung inflammation induced by influenza virus infection through inhibiting the NF-κB signaling pathway', *Influenza and other Respiratory Viruses*, 11(5), pp. 457–463. doi: 10.1111/irv.12459.

Yoon WJ, Lee NH, Hyun CG. 2010. Limonene suppresses lipopolysaccharide induced production of nitric oxide, prostaglandin E2, and pro-inflammatory cytokines in RAW 264.7 macrophages. *J Oleo Sci*. 59: 415-21. doi: http://dx.doi.org/10.5650/jos.59.415

Yuan, S. (2013) 'Drugs to cure avian influenza infection - multiple ways to prevent cell death.', *Cell death & disease*. Nature Publishing Group, 4(10), p. e835. doi: 10.1038/cddis.2013.367.

FIGURE LEGENDS

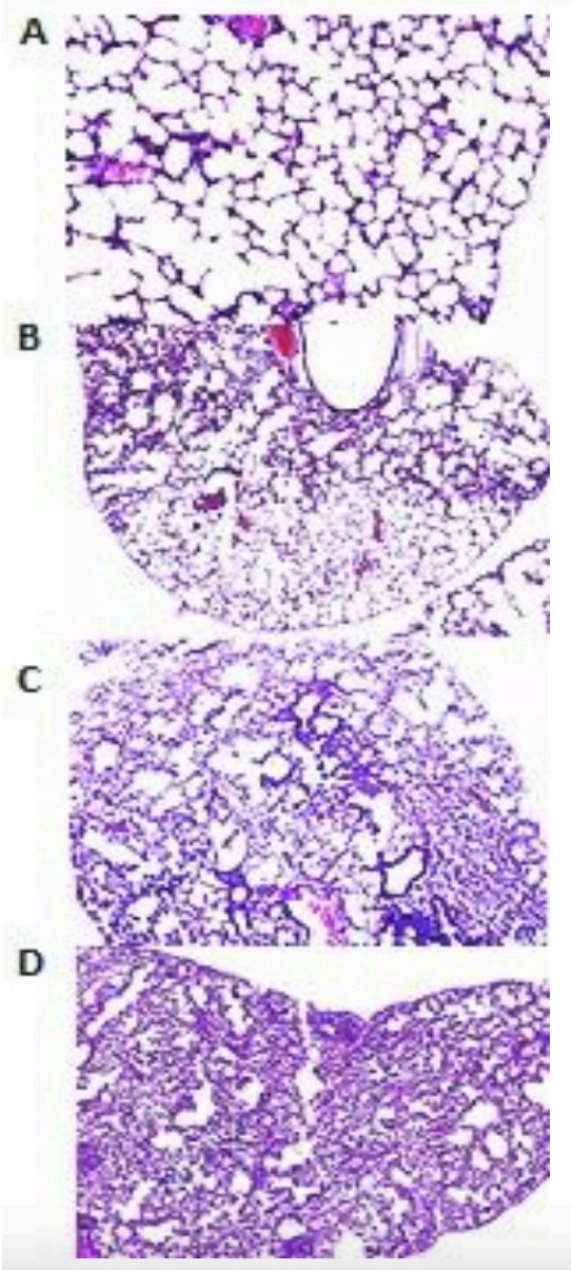

Figure 1: Histopathological lung alterations of A(H1N1)2009 Influenza Virus infection through viral passages. Lung sections from: non-infected animals (A), and from first, second and third (B, C, D) viral passage, respectively. Magnification 10x.

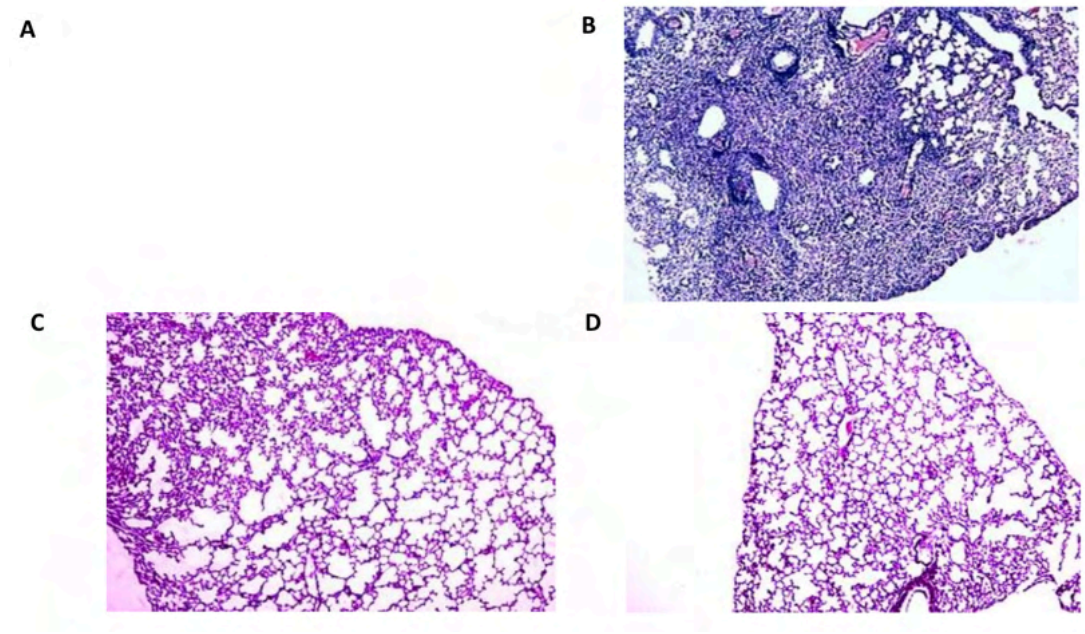

Figure 2: Pulmonary histopathology of lungs from animals with the different treatments. A. Non-infected mice (V_{AH1N1}+V_{PPEO}) B. Infected mice instilled with 5% tween 80 saline solution (AH1N1+ V_{PPEO}). C. Infected group with therapeutic administration of *PPEO* (AH1N1+ PPEO). D. Infected group with prophylactic administration of *PPEO* (PPEO+AH1N1). Magnification 10x.

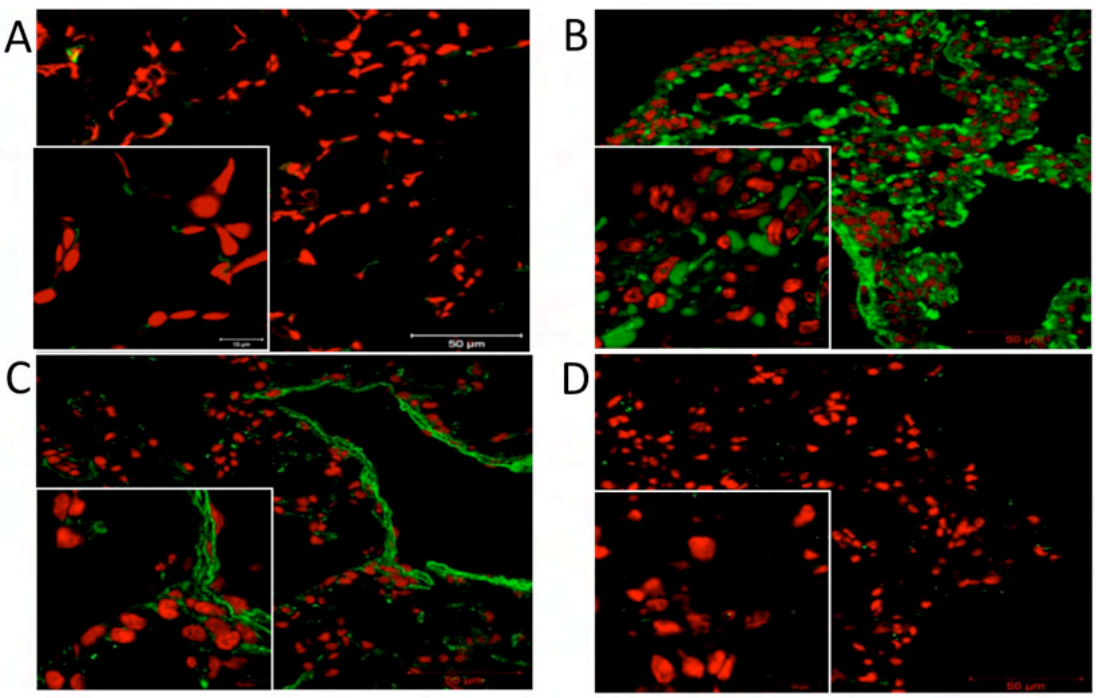

Figure 3: Immunodetection of NF-κB in lungs from animals with the different treatments. Lung sections from animals of different treatment groups are shown. A. Non-infected group without treatment (V_{AH1N1}+V_{PPEO}); B. Infected group without treatment; (AH1N1+ V_{PPEO}). C. Infected group with therapeutic administration of PEO (AH1N1+ PPEO), D. Infected group with prophylactic administration of *PPEO* (PPEO+AH1N1).