Effect of different herbal preparations on experimental viral respiratory complex of broilers: clinical, pathological and ciliary activity aspects

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Implication for health policy/practice/research/medical education: The outcome of the study may be helpful for increasing veterinarians’ knowledge to choose proper therapeutic agents in confronting broiler respiratory viral complex.


Introduction
Viral diseases of respiratory tract affect commercial poultry farms worldwide. Low pathogenic avian influenza viruses (AIVs) such as H9N2 have become enzootic in poultry in many Middle Eastern countries. Despite long-term vaccination programs against H9N2 in some countries, economic losses due to this virus in poultry industry are noticeable and should be considered as a continuous threat (1). The H9N2 virus has the potential to cause severe respiratory disease in combination with other viruses or bacteria including the Newcastle disease virus, infectious bronchitis virus (IBV) and Mycoplasma gallisepticum (MG) (2). The avian infectious bronchitis (IB) virus continues to be one of the most economically...
damaging diseases in chickens despite intensive control (3). High prevalence of mixed infections of AIV-H9N2 and IBV has been reported in field studies (4, 5) which shows a potential role of IBV or even IB live vaccines in exacerbating the clinical manifestation of H9N2 infection in broilers (6).

There are no specific treatments for viral diseases and antiviral drugs are not ordinarily used in poultry industry. Antibacterial drugs may be used to prevent and treat secondary bacterial infections. On the other hand, there are a number of commercially available herbal preparations which are used in many countries as symptom relieving agents in respiratory tract infections. However, rational recommendation of these drugs for management of respiratory diseases in poultry is controversial due to lack of information from well-designed experimental trials.

Essential oils have long been recognized for their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (7-9). The chemical composition and concentration of essential oils are variable. Essential oils are generally used as blend with a carrier oil or in combination with other plant oils in the feed to enhance the productive performance of birds (10). In the majority of circumstances most of the commercial medicines are made by mixing three or more types of essential oils. Few studies have evaluated the use of essential oils in respiratory diseases of chickens. Barbour et al demonstrated that Mentofin® (a herbal product containing 10% eucalyptus oil, 10% menthol, 33% liquid builders and 47% saponins) had a healing effect against MG/H9N2 infection in broilers (11,12). It has been shown that Zataria multiflora essential oil has a positive effect on reducing H9N2 AIV replication, decreasing clinical signs and improving the performance in broilers (13). In a research study by Rehman et al, Mentofin® treated chickens showed higher antibody titer against ND virus vaccine as compared to untreated groups (14). Effect of Mentofin® on MG clearance from the tracheal epithelium has also been shown by Kahaya et al (15). Moreover, the positive effects of volatile oils administrations on immune response of birds to vaccines have been reported (16-18).

Menthol, a monoterpenoid synthesized by plants from Mentha genus (19), and menthol containing agents as well as mucoactive drugs such as bromhexine, which is a derivate of the Adhatoda vasica plant industry (20), are widely used to alleviate bronchitis or as a therapeutic option for mucus alterations, including hypersecretion in the treatment of respiratory diseases of poultry.

On the contrary, there are frequent oral claims from clinicians that treatment of viral respiratory diseases in broiler chickens which is carried out mostly through the administration of respiratory symptom relieving agents, not only doesn't have any positive effects on mortality rate but also may deteriorate the condition. Moreover, treatment with these agents is linked to significant expenses. In fact, knowledge on the effectiveness of these therapeutic modalities is scarce. The mechanism of action of these drugs is also unknown in birds. Thus, in an attempt to further investigate the outcome of using three available respiratory symptom relieving compounds including Mentofin®, Aromax® and Kimia Mentholhexine® in an experimental complex respiratory disease (H9N2+IB) in broiler chickens, this study was performed. The study was based on clinical, histopathological and ciliary activity evaluations.

Materials and Methods
Challenge viruses
H9N2 AIV subtype (A/chicken/Iran/772/1998/H9N2) and variant IBV isolate (793/B serotype IBFIBV32) were used as challenge viruses in the present study. Each viral stock was propagated separately by injecting the allantoic cavity of 9-day-old embryonated chicken eggs and subsequently titrated according to the Reed and Muench method, to determine 50% embryo infectious dose (EID$_{50}$) (21). H9N2 virus was used in a dose of 10$^3$ EID50/bird (50 μL intranasally by nasal instillation and 50 μL through eye drop) and IBV virus in a dose of 10$^4$ EID50/bird IBV (50 μL intranasally by nasal instillation and 50 μL through eye drop).

Chickens
A total of 175 one-day-old commercial broiler chickens (Hubbard) were purchased and used for this experiment. The birds were randomly divided into different groups. To prevent cross-contamination throughout the course of the experiment, each group was housed in an isolated room under strict biosecurity conditions. All chicks in this experiments were not vaccinated. Birds had free access to feed and water. Diet formulation and environmental factors were adjusted in accordance with the Hubbard company rearing manual. This study was carried out in accordance with ethical guidelines of the Institutional Animal Care and Use Committee, which set by the European convention (No. 123).

Experimental design
In this experiment, broiler chickens (described above) were assigned at random into 5 groups (35 birds/group). Two groups of birds were not treated and one group was served as challenged group (positive control: PC; n=35) and another group was assigned as non-challenged negative control (NC; n=35). At 25 days of age, the birds in PC and treatment groups (G1-G3) were challenged. To ensure that infection had occurred, the birds in each group were examined twice daily for clinical signs of the disease. Administration of herbal preparations into treatment groups was started after manifestation of clinical sings [from day 2 post inoculation (PI)] and continued for 5 consecutive days. The birds in treatment groups were
treated as follows: G1) Mentofin® (10 % eucalyptus oil, 10% menthol, 33% liquid builders and 47% saponins) 200 ml/1000 ml drinking water ;( EWABO, Germany); G2) Aromax® (Eucalyptus oil, Mint oil, L-Menthol, Thyme oil) (XVet, Germany) 200 mL/1000 ml drinking water twice daily; G3) Kimia Mentholhexine® (Bromhexine 1%+ menthol) (Kimiafaam Pharmaceutical Co. Tehran, Iran) 1/1000 v/v in drinking water. Dosage of each compound was based on the manufacturer's recommendations. Body weight gain and feed consumption of chickens and clinical signs of the disease were recorded daily from day 25 to the end of study period (12 days post infection).

Sample collection and preparation
On days 1, 3, 6 and 12 post challenge, five birds from each group were euthanized with intravenous injection of sodium pentobarbital (50 mg/kg) and necropsied immediately. Gross lesions were recorded and the samples of each bird (5 birds) were collected for histopathological and ciliostasis evaluation at days 1, 3 and 6 post inoculation as follows: Tracheal (lower part, near to tracheal bifurcation) samples were collected in 10% neutral buffered formalin and after dehydration, embedded in paraffin. Serial sections (5-μm) were cut for hematoxylin and eosin (H&E) as well as periodic acid–Schiff (PAS) staining. Three slides were prepared from each sample of each bird for each H&E and PAS staining. Each slide contained 3 sections cut. In addition, tracheal sections from upper, middle and lower parts of trachea were also harvested from each of scarified birds per group at days 1, 3 and 6 post challenge for ciliostasis assays.

Determination of clinical severity
To evaluate severity, clinical scoring was used. Clinical signs were scored as described previously by Jackwood et al (1: normal; 2: watery eyes or mucus in the nares; 3: watery eyes and mucus in the nares; 4: watery eyes, mucus in nares and tracheal rales) (22).

Lesions of the tracheas, lungs and air sacs were scored for gross severity in 5 birds of each group. The gross lesions in trachea (mucin exudates and cast), syrinx (tubular cast), air sacs (airsacculitis) and lung (congestion and bronchial cast) were scored. Briefly, respiratory organs were scored altogether on scale of 1 to 4, as 1: no lesions, 2: mild or slight lesions, 3: moderate lesions, and 4: severe lesions. Dead birds in each group were also necropsied and qualitatively evaluated.

Histopathological evaluation
The histological findings were graded on a scale of 1 (normal) to 4 (severe) (22) in 5 randomly selected microscopic field of each tracheal section for the following variables: epithelial hyperplasia, lymphocyte infiltration and the epithelial deciliation. The number of goblet cells in the epithelium of trachea was counted in 5 microscopic field in each of the PAS stained sections. These values were then averaged.

Ciliostasis assay
In order to remove mucin from the tracheal epithelial surface, tracheal samples were placed in Hank's balanced salt solution containers. Three thin rings from the upper and lower parts of the trachea and four from the middle part were prepared by surgical blade. The rings were placed separately in tissue culture micro plates containing Dulbecco’s modified eagle medium and were shaken gently at 37°C for few minutes. The ciliary movement was then assessed microscopically and scored on scale from 0 (100% ciliary activity) to 4 (total ciliostasis) (23). These scores were then averaged to give a final score.

Data analysis
Statistical analysis was performed using SPSS software version 22. All data were expressed as the mean ± standard deviation (SD) and analyzed by parametric or nonparametric statistics including one-way ANOVA followed by Tukey's multiple comparison, Mann-Whitney U test and Kruskal-Wallis one-way analysis of variance where appropriate. For all cases differences were considered significant at P < 0.05

Results
Clinical severity evaluations
All of the birds in negative control group were scored as normal for clinical signs, gross and histopathological parameters through the experiment and no mortality was observed in this group.

Mild respiratory signs including watery eyes or mucosa in nares were observed in most of the birds in challenged groups regardless of treatment which appeared from day 2 PI. Respiratory signs were observed from day 3 to day 9 PI including nasal discharge, rales and excessive lacrimation with the most severe signs appeared on day 6 PI. Table 1 shows that clinical signs scores for the treated and challenged groups were statistically higher than NC group while no significant differences were observed between PC and treated groups at days 1, 3, 6 and 9 PI. In addition, clinical scores were statistically the same among treated groups on these days. On day 12 post challenge, no differences were observed among birds alive in NC, PC and all treated groups.

Mortality rate in PC group was 8.6% while G3 (bromhexine 1%+ menthol treated) group appeared to have the highest mortality rate (28.6%) followed by G1 (Mentofin® treated group; 25.7%) and G2 (Aromax®; 22.8%) groups (Table 1). In PC and treatment groups death was initiated from day 4 and peaked during day 6 to 8 PI. Death occurred until day 9 in G3 and G1 groups. No differences were observed in body weight among groups at the end of the experiment (Table 1). All challenged
Table 1. Body weight, FCR, scores of clinical signs and mortality rate in different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Clinical signs severity (Mean±SD)</th>
<th>Mortality No. (%)</th>
<th>Body weight (g) (Mean±SD)</th>
<th>FCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>NC</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PC</td>
<td>1.4±0.52  a</td>
<td>2.7±0.67a  b</td>
<td>3.2±0.42  a</td>
<td>2.8±0.79  a</td>
</tr>
<tr>
<td>G1</td>
<td>1.4±0.52  a</td>
<td>2.6±0.69  b</td>
<td>3.5±0.71  a</td>
<td>3.1±0.73  a</td>
</tr>
<tr>
<td>G2</td>
<td>1.4±0.52  a</td>
<td>2.8±0.42  a</td>
<td>3.6±0.69  a</td>
<td>3.2±0.79  a</td>
</tr>
<tr>
<td>G3</td>
<td>1.4±0.52  a</td>
<td>2.7±0.82  a</td>
<td>3.6±0.69  a</td>
<td>3±0.94  a,b</td>
</tr>
</tbody>
</table>

1 = normal, 2 = watery eyes or mucus in the nares, 3 = watery eyes and mucus in the nares, and 4 = watery eyes, mucus in the nares and tracheal rales; DPI: days post infection; Groups: NC: Negative control, PC: Positive control, G1: Mentofin®, G2: Aromax®, G3: Bromhexine 1% + menthol; N: number; FCR: feed conversion ratio.
Different superscript letter in a column indicates significant difference (P < 0.05).

Table 2. Scores of gross lesions (Mean±SD) in different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td>NC</td>
<td>0*</td>
<td>0*</td>
<td>0*</td>
<td>0*</td>
<td>0*</td>
<td>0*</td>
</tr>
<tr>
<td>PC</td>
<td>2±0.707  b</td>
<td>2*</td>
<td>1±0*</td>
<td>1*</td>
<td>2±0.707  b</td>
<td>1*</td>
</tr>
<tr>
<td>G1</td>
<td>2±1  a</td>
<td>2*</td>
<td>0*</td>
<td>0*</td>
<td>1±0.707  b</td>
<td>1*</td>
</tr>
<tr>
<td>G2</td>
<td>2±0.707  b</td>
<td>2*</td>
<td>0*</td>
<td>0*</td>
<td>1±0.707  b</td>
<td>1*</td>
</tr>
<tr>
<td>G3</td>
<td>2±1  a</td>
<td>2*</td>
<td>0*</td>
<td>0*</td>
<td>1±0.707  b</td>
<td>1*</td>
</tr>
</tbody>
</table>

1 = no lesions, 2 = mild or slight lesions, 3 = moderate lesions, and 4 = severe lesions, Groups: NC: Negative control, PC: Positive control, G1: Mentofin®, G2: Aromax®, G3: Bromhexine 1% + menthol. A: tracheal congestion; B: tracheal exudates; C: cast formation in trachea; D: Cast formation in tracheal bifurcation; E: lung congestion; F: pleuropneumonia; G: airsacculitis; SD: standard deviation.
Different superscript letter in a column indicates significant difference (P < 0.05).
groups had higher FCR than NC group.

**Gross lesions severity**
Significant lesions were observed in gross pathological examination of all challenged groups compared to NC group through the experiment. Gross lesions including tracheal exudates, congestion of lung and airsacculitis were observed. Although the gross scores in all treated groups were higher than PC group throughout the experiment, the data were not statistically different. The results are shown in Table 2.

Qualitative evaluation of gross lesions in dead birds of all treatment groups showed that all lesions were more severe in these groups than dead birds of PC group and also involved more birds. Lesions such as cast formation in trachea and syrinx (Figure 1A), tracheal congestion, tracheal exudates, airsacculitis (Figure 1B), congestion of lungs and pleuropneumonia were observed in most of the dead birds in treated groups. Cast formation in tracheal bifurcation and tracheal congestion were the most prominent lesions observed in dead birds of all groups. Tracheal cast/plug was extended to primary bronchi in some cases (data not shown).

**Histopathological evaluation**
The main lesions observed in challenged groups (H & E staining) included loss of cilia in the epithelial cells, epithelial hyperplasia and lymphocyte infiltration into the mucosa of trachea which were mainly initiated from day 3 PI. The nature of the histological lesions and the severity and persistence of lesions did not differ among challenged (PC + treatments groups) groups in all sampling times. The most severe lesions were observed on day 9 PI (Table 3). In the tracheas (PAS staining), significant goblet cell hyperplasia of surface epithelial cells was observed in all treatment groups on days 3 and 6 PI compared to PC and NC groups (P<0.01) (Figure 2). No significant differences were observed in this parameter among treatment groups (Figure 3).

**Ciliostasis**
Although ciliostasis was observed in tracheal samples of birds in all challenged groups, statistically significant differences in the ciliary activity among challenged groups,

![Figure 1. Cast formation at the tracheal bifurcation of challenged birds (A). Slight cloudiness and foamy content of air sac membrane (B).](image1)

![Figure 2. Tracheal goblet cell changes in broiler chickens of different groups on day 6 post challenge with AIV-H9N2 and IB viruses (PAS staining; magnified 40x). Marked goblet cells and goblet glands hyperplasia as well as degeneration of goblet cells are observed in treatment groups. (A): Positive control [Goblet cells (arrow)], (B): Negative control, (C): Mentofin® [Goblet glands (arrow)], (D): bromhexine1%+menthol [Degeneration of goblet cells (arrowhead)], (E): Aromax® [Goblet glands (arrow)].](image2)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Histopathology score* (Mean±SD)</th>
<th>Days post infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>NC</td>
<td>1.1±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PC</td>
<td>1.1±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4±0.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G1</td>
<td>1.1±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.6±0.59&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G2</td>
<td>1.1±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4±0.719&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G3</td>
<td>1.1±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.6±0.703&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*The histopathology score was based on epithelial hyperplasia, lymphocyte infiltration, and epithelial deciliation with 1 being normal and 4 being severe. Groups: NC: Negative control, PC: Positive control, G1: Mentofin®, G2: Aromax®, G3: Bromhexine 1% + menthol. Different superscript letters in a column indicates significant difference (P < 0.05). SD: Standard deviation.

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even among treated birds and PC, were not detected in different sampling days (Table 4).

**Discussion**
Symptom relieving agents are widely used in most of the poultry respiratory infections regardless of etiology (viral or bacterial) with little evidence of effectiveness. Many of these drugs are combined products of different herbal derived aromatic volatile substances such as menthol, thymol, eucalyptol and cineol. Menthol has especially attracted much interest in poultry industry and diverse commercial respiratory symptom relieving compounds containing menthol are promoted for use in respiratory infections. However, the exact clinical effectiveness of using these drugs in birds is still debated. In this study, the effects of administration of three available menthol containing medicines on clinical severity and some histopathological aspects were evaluated in broilers infected with H9N2 and IB viruses.

As our first finding, clinical manifestation of H9N2-AIV/IBV infection was limited to respiratory signs in all challenged groups regardless of treatment. Mild to moderate clinical signs prolonged to day 9 post challenge. Similar clinical signs were previously reported in mixed H9N2-AI/IBV infection of broilers (24,25). The clinical signs and gross lesions resulting from the dual infection in PC group are similar to but generally milder than those seen in field cases. Moreover, the mortality rate in PC group was only 8.6 % while higher mortality rate has been reported in field conditions (4,26,27). This difference can be attributed to inadequate management and environmental conditions (poor ventilation, high density, high ammonia levels and too low or high relative humidity) and additional pathogens encountered in the field, exacerbating any disease which has been brought about.

Contrary to expectation, treatment with herbal drugs or mucolytic agent not only did not decrease the severity of clinical signs or gross pathological lesions in challenged-treated birds (G1-G3) but also increased the mortality rate in these groups which was similar to those reported by Faghihzadeh et al who reported high mortality in experimentally infected (H9N2+IBV+E. coli) broilers given Bronchimax® or bromhexine compare to PC group (25). These data do not agree with other workers (11) who reported that Mentofin® treatment reduced the mortality in challenged (H9N2 AIV + IBV +MG) and unchallenged broilers.

Treatment with herbal compounds had no effect on body weight and FCR in challenged chickens. Consistently in a study reported by Rehman et al. (2013), Mentofin® did not show any effect on FCR and weight gain of treated broilers vaccinated with Newcastle disease vaccine (14). In contrast, Sultan et al demonstrated better performance (lower FCR and higher body weight) in H9N2 infected broilers treated with intermittent administration of Mentofin® (28).

As it was mentioned in the result section, gross lesions in euthanized birds of challenged groups were also limited to respiratory system with no significant differences among groups. Tracheal exudates, airsacculitis, tracheal congestion as well as bronchial casts/plugs formation mostly extended to the lower bronchi were the main lesions observed in mixed infection of H9N2 –AI and IB viruses in dead birds of challenged groups which were in accordance with previous reports (24,25). According to necropsy findings in dead birds of all challenged groups, it seems that mucoid or fibrinonecrotic cast/plug formation especially in tracheal bifurcation which is observed in mixed infection of H9N2/IB viruses (24,26,29) is the main cause for partial to complete blockage of trachea.

<table>
<thead>
<tr>
<th>Ciliary activity score (Mean±SD)</th>
<th>Treatment groups</th>
<th>DPI</th>
<th>1</th>
<th>3</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>0</td>
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<td>2.36±0.167&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>G2</td>
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<td>2.24±0.251&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5±0.1000&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>G3</td>
<td>0</td>
<td>2.14±0.0194&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4±0.141&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

<sup>a</sup> The ciliary movement scored on scale from 0 (100% ciliary activity) to 4 (total ciliostasis). Groups: NC: Negative control, PC: Positive control, G1: Mentofin®, G2: Aromax®, G3: Bromhexine 1% + menthol. Different letters in each column indicate significant differences between groups tested at the same time point post infection; SD: standard deviation.
Mucosa (33). Ciliostasis was observed in all challenged groups of this study regardless of treatment. Administration of bromhexine1% + menthol caused higher death (28.6%) compared to G1 and G3 groups. Rheological study of tracheobronchial secretions of chicken treated with various mucolytic drugs revealed that cilia from healthy birds medicated with bromhexine, required greater force to displace mucus compared to untreated birds (34). According to this fact, decreasing mucus motion following mucolytic activity of bromhexine may be involved in worsening severity of the clinical manifestation of the disease in the chickens of group G3.

Conclusion

Overall, our results indicated that use of herbal medications, which is very common in the symptomatic treatment of respiratory disease in poultry industry, aggravates mortality and deteriorates the conditions and should be used cautiously and after proper clinical judgement. It appears that hypersecretion of mucus along with ciliary incompetence causes mucus stagnation followed by tracheal or bronchial obstruction and death. These findings necessitate cautious use of these products. Nevertheless, further work is required.

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Authors’ contributions

DH and MN had major roles in hypothesis making, preparation of the manuscript and data interpretation. AM committed data acquisition and sampling. BZ and RG had major roles in running experiments. All authors have read and approved the manuscript.

Conflict of interests

All authors declare that no conflict of interest exist.

Ethical considerations

This study was carried out in accordance with ethical guidelines of the Institutional Animal Care and Use Committee, which set by the European convention (No.123; Appendix A). The project was confirmed by the ethical committee of Shiraz University (Code number: 95GCU2M163723).

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Herbal preparations and experimental viral respiratory complex of broilers


