Safety and Antiviral Activity of essential oil Against Avian Influenza and NewCastle Disease Viruses

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ABSTRACT

The objective of this research is to determine the safety and virucidal activity of different levels of Eucalyptus-peppermint essential oil (Mentofin®) against Avian Influenza Virus (AIV) and NewCastle disease virus (NDV) in presence and absence of 1 % skim milk. All five concentrations of Mentofin® (2.78x10-3, 2.78x10-2, 2.78x10-1, 2.78, 13.9, and 27.8%) were safe when administered in 0.1 ml/chick embryo, resulting in 100% survival of embryoes. The virucidal activity against AIV started at the Mentofin® concentration of 2.78x10-1 % and 30 minutes contact time at room temperature, only in the absence of skim milk. The complete virucidal activity in presence and absence of skim milk, against AIV and NDV occurred at the 2.78%, and 13.9%, respective concentration of Mentofin®, using the 30 minutes-contact time. The need for a higher concentration of essential oil to inactivate the NDV (13.9%) in comparison to a lower concentration needed to inactivate the AIV (2.78%) is discussed at the level of differences in the structure of the two viruses.

INTRODUCTION

The research in the use of natural essential oils as antimicrobials for treatment of certain animal diseases is of paramount interest to scientists and the poultry industry.[1,2] This is due to the new pressure resulting from banning the use of synthetic drugs in poultry and other animals[3] due to the health hazards that could occur in humans consuming residues of synthetic drugs present in meat or eggs, and due to induction of emergence of resistance to extensively used drugs in pathogens. In addition, the use of essential oils containing more than one active ingredient, working in synergism, hinders the ability of pathogens to develop resistance,[4] and thus ending in most of the conditions in the

inactivation of the microorganisms and in survival and better performance of the host.

The purpose of this research is to study the safety and antimicrobial activity of Mentofin®, containing the essential oils of the leaves of Eucalyptus and Peppermint plants, against two important pathogens, causing economic diseases in poultry, namely, the Avian Influenza and NewCastle Disease Viruses.

MATERIALS AND METHODS

A. Mentofin® concentrations

The recommended use of Mentofin® (EWA-BO, Wietmarschen, Germany) in drinking water for poultry is at the level of 0.25 ml/ kg body weight. The volume of blood in a chicken per 1 kg body weight is about 90ml, resulting in a concentration level of Mentofin® in the blood of a treated chicken of around 2.78x10-2%, assuming a 100% absorption. This 2.78x10-2 % concentration will be considered in this research as the X level, and the design of the experiment will

Table 1: *Tabulated experimental design for the in vitro-antimicrobial testing of Mentofin® at different concentrations against Avian Influenza and Newcastle Disease viruses in presence and absence of 1% skim milk*

Mentofin® concentration (%)	30 minutes contact with	1% skim milk	No. of chicken embryoes used to propagate the AI or ND viruses
X/10 ($2.78x10^{-3}$ %)	AI virus	Absence	2
	AI virus	Presence	2
	ND virus	Absence	2
	ND virus	Presence	2
X ($2.78x10^{-2}$ %)	AI virus	Absence	2
	AI virus	Presence	2
	ND virus	Absence	2
	ND virus	Presence	2
10X ($2.78x10^{-1}$ %)	AI virus	Absence	2
	AI virus	Presence	2
	ND virus	Absence	2
	ND virus	Presence	2
100X (2.78%)	AI virus	Absence	2
	AI virus	Presence	2
	ND virus	Absence	2
	ND virus	Presence	2
500x (13.9 %)	AI virus	Absence	2
	AI virus	Presence	2
	ND virus	Absence	2
	ND virus	Presence	2
Positive control (No Mentofin® contact)	AI virus	Absence	2
	ND virus	Absence	2
Negative control	---*	Absence	3
		Total	47

**Embryoes not injected with any of the viruses*

evaluate the antimicrobial activity of Mentofin® at different concentrations namely, X/10, X, 10X, 100X, and 500X.

B. Experimental Design

The five concentrations of Mentofin® will be prepared and used each in vitro against the Avian Influenza (H9N2 subtype) and NewCastle Disease virus in the presence and absence of organic matter of skim milk (1%), as presented in Table 1. The experimental design included a 30 minutes contact time between the Mentofin® components at X/10 - 500X levels and each of the two viruses, at room temperature. The density of each of the two viruses used in contact was 4x107 particles. This contact was performed in presence and absence of 1 % skim milk to study the effect of the presence of organic matter in interfering with the virucidal activities of Mentofin®. At the end of the contact time between the different concentrations of Mentofin® and each of the two viruses, in presence and absence of skim milk, the survival of the two viruses was checked by injecting the reactants in 0.1 ml volume in live 10 day-old chick embryoes, using duplicate embryoes for each treatment, as shown in the last column of Table 1. Positive controls were included in the design, in which AIV and NDV were deprived of contact with Mentofin® and with skim milk, and were injected each in duplicate chick embryoes. In addition, a negative control of three embryoes was included, left with no viral inoculation, to proof their absence of contamination by AIV and NDV. The differently inoculated embryoes were re-set in the incubator at 99°F and 85% humidity for a period of 3 days, followed by candling to check the viability of the embryoes, in an attempt to evaluate the safety of different levels of Mentofin® on the embryoes. After candling and recording of the viability of each embryo, the embryonated eggs were put in the fridge at 5°C to coagulate the blood in the embryoes, thus preventing the contamination of the allantoic fluid with blood of the embryoes. Individual collection of the allantoic fluid from each embryo is concluded, and the proof of the ability of the virus to survive the Mentofin®-treatment and be able to propagate in the embryonic cells was done by performing the Hemagglutination (HA) test on the allantoic fluid.

Table 2: *Anti-Avian Influenza viral activity by different concentrations of Mentofin® in the presence and absence of 1% skim milk*

Mentofin® concentration (%)	1% skim milk	Virucidal activity* of Mentofin®
X/10	Present	None
($2.78x10^{-3}$ %)	Absent	None
X	Present	None
(2.78x10-2 %)	Absent	None
10X	Present	None
(2.78x10-1 %)	Absent	100%
100X	Present	100%
(2.78%)	Absent	100%
500x	Present	100%
(13.9 %)	Absent	100%
Positive control	Absent	Not Applicable (presence of replicating virus)
Negative control	Absent	Not Applicable (absence of replicating virus)

** The virucidal activity of Mentofin® is reported as none, meaning presence of hemagglutination due to survival of replicating virus, and 100% virucidal activity, due to complete absence of hemagglutinating virus in the allantoic fluid of the embryoes.*

***Table 3**: Anti-Newcastle Disease viral activity by different concentrations of Mentofin® in the presence and absence of 1% skim milk*

Mentofin® concentration (%)	1% skim milk	Virucidal activity* of Mentofin®
X/10	Present	None
($2.78x10^{-3}$ %)	Absent	None
X	Present	None
(2.78x10-2 %)	Absent	None
10X	Present	None
(2.78x10-1 %)	Absent	None
100X	Present	None
(2.78%)	Absent	100%
500x	Present	100%
(13.9 %)	Absent	100%
Positive control	Absent	Not Applicable (presence of replicating virus)
Negative control	Absent	Not Applicable (absence of replicating virus)

** The virucidal activity of Mentofin® is reported as none, meaning presence of hemagglutination due to survival of replicating virus, and 100% virucidal activity, due to complete absence of hemagglutinating virus in the allantoic fluid of the embryoes.*

The performance of the HA test was done by preparation of 1% suspension of washed red blood cells (RBC) of chicken, and reacting each allantoic fluid with an equal volume of 50µl of 0.1% RBC at room temperature for a period of 45 minutes. The presence of complete hemagglutination, and prevention of precipitation of the RBC at the bottom of wells of the round-bottom Micro-Titer plates, is indicative of the presence of the surviving replicated virus in the allantoic fluid. Controls were included in the Micro-Titer plate in which an equal volume of 0.1% RBC and saline (1:1 v/v) were included in duplicate in the wells, to proof that the RBC will settle to the bottom of the wells after 45 minutes of their introduction to the wells of the Micro-Titer plate.

RESULTS AND DISCUSSION

Safety

All embryoes exposed to Mentofin® concentrations of X/10- 500X survived, as shown in the candling performed after three days of delivery of the reactants (Mentofin®-virus, in presence or absence of skim milk). In addition, all positive and negative controls survived too. This is indicative that the Mentofin® itself, the two viruses, and the skim milk have no killing effect on the embryoes.

Anti-Avian Influenza Viral Activity by Mentofin®

Table 2 shows the virucidal activity by different concentrations of Mentofin® against the Avian Influenza virus, in the presence and absence of 1% skim milk.

The complete virucidal activity of Mentofin® against the Avian Influenza virus started to appear at 10X concentration and above. There was an absence of a complete virucidal activity against H9N2-Avian Influenza virus by Mentofin® used at X/10 and X level in presence and absence of skim milk. At Mentofin® concentration of 10X, a complete virucidal activity against Avian Influenza occurred in the absence but not in the presence of skim milk, which is most likely due to the neutralizing effect of organic matter in skim milk to the Mentofin® at this 10x level. Previous studies have confirmed the interfering nature of organic matter with the disinfectant activities against microorganisms.[1,2] When the Mentofin® concentration was raised to 100X and 500X there was a complete virucidal

effect against Avian Influenza Virus, even in the presence of 1% skim milk. The concentration of disinfectants plays a major role in inactivation of organisms.[1,2] This data shows that even with a short contact time of 30 minutes, the virucidal activity started to appear at 10X level. The treatment of poultry with the Mentofin®, at the manufacturer recommended level, and for certain specified days (usually ≥ 3 days), will elongate the contact time between the Mentofin® components and the Avian Influenza Virus in the host, which is expected to raise the virucidal effect of Mentofin® against the Avian Influenza Virus, and possibly at lower concentrations of this preparation. The contact time of disinfectants and microorganisms is important in the inactivation process.[1,4]

Anti-Newcastle Disease Viral Activity by Mentofin®

Table 3 shows the virucidal activity by different concentrations of Mentofin® against the Newcastle Disease Virus (NDV), in the presence and absence of 1% skim milk. The ND Virus seems more resistant than the AI Virus, in which observation of complete virucidal effect against NDV occurred at a higher concentration of Mentofin® namely at 100X, and in the absence of organic components of skim milk. Reaching to a complete virucidal effect against NDV, in the presence of organic matter, needed even a higher concentration of Mentofin® namely, the 500X. The composition nature of the NDV is significantly different from the AIV. Actually, the NDV needs a fusion protein for providing its penetration into the host cell,[5] which could be a resistant protein to components of the essential oil, while the Influenza virus penetration is by a receptor – mediated endocytosis mechanism,[6] which could be affected by the effect of the essential oil on the attachment, by affecting the configuration of the hemagglutinin protein. It is worth noting that the data presented in Table 3, is obtained by an in-vitro short contact time of 30 minutes, at room temperature. Again, the in vivo- reaction of Mentofin® with NDV occurs at the chicken temperature (~ 41.2 oC), and for a longer time of the recommended treatments (usually ≥ 3 days). This will allow for a longer contact time and at a higher temperature level, which could raise the virucidal activity of Mentofin® against Newcastle disease Virus, possibly at lower concentrations of Mentofin®.

In conclusion, an in vitro- contact time of Mentofin® of 30 minutes at room temperature showed a virucidal effect in both AIV and NDV, with more susceptibility of AIV compared to NDV to this essential oil preparation. The presence of organic matter at 1% level is expected always to interfere with the antiviral activity, which required higher levels of Mentofin® to overcome this interfering effect and to maintain essential oil's virucidal effect.

Future investigation should evaluate the antiviral effect of Mentofin® at the normal temperature of the chicken (41.2°C), and for longer contact periods extending up to the end of the usual treatment periods recommended by the manufacturer of this preparation. This in vitro- contact of Mentofin® with viruses should be done in the presence and absence of major interfering components of the chicken serum, such as the albumin.

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