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Studies on the antiviral activity of some herbal plants extract against isolated duck hepatitis virus in infected ducklings.

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ABSTRACT

Duck hepatitis virus (DHV) is a fatal, rapid spreading viral infection of young ducklings that causes hepatitis and has a high mortality rate if not managed. In the present work, fifteen previously identifiedDHAV3 isolates using conventional RT-PCR. RT-PCR were taken from 29 positive isolates and cultured on ECE. All isolates were identified using conventional RT-PCR. RT-PCR was used for detection and subtyping of DHAV using specific pairs of primers to amplify the UTR and VP1 gene respectively. These isolates were used in experimental infection. The efficacy of three herbal substances included Ment, Neem and Curcumin against DHAV3 infection was evaluated in ten-day-old Pekin ducklings inoculated i/m with 10^6 EID_{50}/0.1ml of DHAV3.Tissues of liver and spleen of inoculated ducklings were examined histopathologicaly. Cloacal swabs were tested using Real Time-PCR to detect virus shedding. The results concluded that the Ment treated group showed the most effective virus reduction followed by Neem treated group then the curcumin treated group.

1. INTRODUCTION

Duck viral hepatitis is a lethal, infectious, and quickly spreading viral disease that affects young ducklings *(OIE, 2018)*. It is caused by three types of viruses. Duck virus hepatitis type I (DVH I) can be caused by duck hepatitis A virus (DHAV) and classified as genus Avihepatovirus, a member of the RNA family Picornaviridae. Duck astroviruses 1 and 2 (DAstV-I and DAstV-II) cause DVH types 2 and 3 respectively (*Wei et al.,2012)*. DHAV occurs worldwide and accounts for more than 80% of mortality in ducklings under 3 weeks *(Li et al., 2013)*. Recently, DHV-1 has been renamed as duck hepatitis A virus (DHAV) and divided into three serotypes designated serotype 1, 2 and 3: Serotype1 (DHAV-1) (the classical serotype) is the most widespread and more virulent serotype worldwide *(Kamomae et al., 2017)*, whereas serotype 2(DHAV-2) was reported in Taiwan and serotype 3 (DHAV-3) in South Korea and *China (Li et al., 2013).*

Despite the vaccine's availability, DHAV-like infections continued to occur, even on farms where DHAV-1 immunization had been introduced. Kim et al. (2007) isolated and identified DHAV-3 in ducklings with DHAV-like illnesses. Because the DHAV-1 vaccination could not provide cross-protection against DHAV-3 infection, a live attenuated DHAV-3 vaccine was produced (Kim et al., 2009). In recent years, DHAV-1 or DHAV-3 infection has grown more common; also, simultaneous co-infection with DHAV-1 and DHAV-3 infection has become more common in Egyptian domestic duck farms (Hassan et al., 2020).

As a result, both DHAV-1 and DHAV-3 vaccinations are strongly advised. However, concurrent administration of both vaccinations may result in antigen interference and adverse effects, reducing the vaccine's protective potency and safety (Vidor, 2007). As a consequence of their broad availability and ease of incorporation into the diet, herbal medicine is gaining prominence in anti-viral research (Abd El-Hamid et al., 2018) .

Therefore, the aim of this study is to evaluate the antiviral activity of herbal plants extract in control of DHAV in infected ducklings.

2. MATERIAL AND METHODS

2.1. Sample collection and preparation:

Fifteen liver samples were aseptically collected from 1-3weekold commercial duck Flocks from different governorates from December 2019 to May2022. Diseased Ducklings were showing typical clinical signs of duck viral hepatitis as described by *(Mansour et al.., 2019)*. Liver samples were prepared under complete aseptic condition according to *(OIE.,2018).* Each sample consisted of (4 pooled liver) manually ground in sterile mortar and pestle to prepare 20% suspension (w/v) in sterile PBS (PH:7.2) solution contain penicillin (1000 IN/ml) & streptomycin (1mg/ml). The suspension subjected to 3 successive cycles of freezing and thawing then sample is clarified by centrifugation at 3000 rpm for 15 minutes at 4°C. The clarified supernatants were transferred into sterile tubes.

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2.2. Methods for extraction of herbal plants:

*Extraction of neem by adding 40gm of azadirachtaindica leaves to 1liter of boiled water and left 6 hours at room temperature then take the extraction of neem 4% and kept at refrigerator. Neem leaves extract was used at dose 50ml/1liter, according to *(Hegazy et al.2013).*

*Extraction of mint by adding 25gm of menthaspicata leaves to 1 liter of boiled water and left 6 hours at room temperature then take the extraction of mint 2.5% and kept at refrigerator. Mint leaves extract was used at dose 50ml/1liter, according to *(Abu Isha et al.2018).*

*Curcuma Longa powder, the powder was purchased from commercial sources and used at dose 200mg/kg, according *(Yadav et al. 2020).*

2.3. Virus isolation on specific pathogen free embryonated chicken eggs (SPF-ECEs):

The supernatant (0.2ml) was inoculated via allantoic cavity into three 9-11 days old embryonated chicken eggs, incubated at 37ºC and candled daily. Uninoculated three eggs were always included as negative controls according to *(Tseng and Tsai 2007).* All embryos were collected excluding non-specific deaths during 1st 24 hours post inoculation and left to be chilled at 4ºC overnight then examined for DHAV characteristic lesions according to *(OIE, 2018).*

2.4. Virus titration:

The 50% egg infectious dose (EID50) was calculated according to *(Reed and Muench 1938).*

2.5. Experimental design:

The study was approved by the ethical committee of research at Benha university of Egypt, Faculty of Veterinary Medicine (Approval number: BUFVTM 08-02-23). Fifty, one day old Pekin ducklings were floor reared and fed on balanced rations to fulfill their nutritional requirement. None of ducklings were vaccinated against DHAV. At the age of ten days, ducklings were divided into 5 groups (table 1):

Group (1): Ten days old ducklings were neither DHAV infected nor treated (-ve control).

Group (2): Ten days old ducklings were infected intramuscular with i/m, $1x10^6$EID50/ML of isolated DHAV3 according to *(Hisham et al.2020)* and not treated with any natural substances (+ve control).

Group (3): Ten days old ducklings were infected i/m with, $1x10^6$ EID50/ML of isolated DHAV3 and treated with curcumin at dose 200mg/kg BWt orally for 6 consecutive days according to *(Yadav et al. 2020)*.

Group (4): Ten days old ducklings were infected i/m with,$1x10^6$ EID50/ML of isolated DHAV3 and treated with 2,5% aqueous extract of Mint (Menthaspicta) at dose 50 ml/Liter orally for 6 consecutive days according to *(Abu Isha et al.2018)*.

Group (5): Ten days old ducklings were infected i/m with, 1x106 EID50/ML of isolated DHAV3 and treated with 4% aqueous Neem leaves extract (Azadirachtaindica) at dose 50 ml/Liter orally for 6 consecutive days according to *(Hegazy et al.2013).*

Infected ducklings were observed daily. The clinical signs and daily mortalities were recorded. Cloacal swabs were taken from all groups at 7th and 14th days post infection. Tissue samples (liver and spleen) were collected from freshly dead and sacrificed infected ducklings for histopathological examination.

2.6. Histopathological examination:

Collected specimens (liver and spleen) were fixed in 10% buffered neutral formalin solution. Five- micron thick paraffin sections were prepared, stained by H&E According to Suvarna et al. (2018) and then examined microscopically.

2.7. Molecular characterization of DHAV isolates which taken from cloacal swabs by Real Time PCR:

A. Extraction of viral RNA using QIAamp Viral RNA Mini Kit:

RNA was extracted from tissue pools using QIA amp viral RNA Mini Kit according to QIAamp Viral RNA Mini handbook (Qiagen, Valencia, USA, Cat. No. 52904**)**.

Oligonucleotide primers sequences

Target gene	Primers sequences	Reference
5'UTR	F. Primer CCTCAGGAACTAGTCTGGA	Fu et al., 2008
	R. Primer GGAGGTGGTGCTGAAA	

B. Preparation of PCR Master Mix according to Quantitect SYBR green PCR kit:

Component	Volume/reaction
2x QuantiTect SYBR Green PCR Master Mix	12.5*µl*
Reverse transcriptase	0.25*µl*
Forward primer (10 pmol)	0.5*µl*
Reverse primer (10 pmol)	0.5*µl*
RNase Free Water	8.25*µl*
Template DNA	3*µl*
Total	25*µl*

C. Thermal profile used for SYBR green Real Time PCR:

Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		
		Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation
50˚C	94˚C	94˚C	50˚C	72˚C	94˚C	50˚C	94˚C
30 min.	5 min.	30 sec.	30 sec.	30 sec.	1 min.	1 min.	1 min.

3. RESULTS

Clinical picture and postmortem lesions:

The examined flocks showed nervous manifestation including ataxia, head drawn back (opisthotonos), imbalance and fall on one side with spasmodically kicking till death. The gross pathological changes of recently dead duckling appeared mainly hemorrhagic liver.

Virus isolation on ECE:

Embryo deaths occurred after 5-7 days of inoculation in the first passage. The second passage showed embryo deaths after 3-5 days. The embryo mortalities were 56% in the first passage and increased up to 84% in the second one. The allantoic fluid was greenish. The collected embryos showed stunting, subcutaneous hemorrhage over the whole body with edema in abdomen and hind limbs and necrotic liver.

The efficacy of herbal plants extracts:

Table 1 shows the experimental design of treatment, mortality rate and protection rate among experimental ducklings.

Group No.	Treatment	Mortality rate	Protection rate
G1*	No treatment (- ve control)	No mortality	-
G2**	No treatment (+ ve control)	90%	-
G3	Curcumin	60%	40%
G4	Mint	10%	90%
G5	Neem	30%	70%

The efficacy of herbal plants extracts (Curcumin, Mint and Neem):

Group (1) (-ve control): The ducklings of this group showed neither clinical signs nor mortalities. The ducklings appeared active and normally eating during the experimental period.

Group (2) (+ve control): Ducklings showed signs of depression and anorexia as early as 3rd day post inoculation (DPI). All ducklings stop moving and showed partially closed eye as well as they suffered from mild to severe neurological signs begin at 4thDPI characterized by imbalance, lethargy and ataxia, falling on their sides and kick spasmodically followed by opisthotonos .Mortalities started on 3rd DPI and were nine ducklings out of ten (90%).Gross examination revealed that ducklings had an enlarged liver with petechial and ecchymotic hemorrhages .The spleen was enlarged with mottling.

Group (3) (Received Curcmin): The ducklings showed signs of depression and anorexia as early as 3rd DPI. The most of ducklings stop moving and partially closed eyes. Neurological signs were noticed only in 4 ducklings prior to death. Mortalities started in the 3rd DPI and were six ducklings out of ten (60%). Grossly, the examined ducklings showed enlarged hemorrhagic liver.

Group (4) (Received Mint): The ducklings showed signs of active, eating and drinking normally and gaining weight throughout the experimental period. Only one duckling was died at 7th DPI (10% mortality rate). Neither neurological signs nor gross lesions in sacrificed ducklings were observed.

Group (5) (Received Neem): The ducklings appeared normally except three ones showed signs of dullness, anorexia, stop moving and partially closed eye till death (30% mortality rate). Neurological signs were noticed only in three ducklings prior to death. Grossly, the examined ducklings showed enlarged hemorrhagic liver.

Results of histopathological examination:

Group (1) (-ve control): Histopathological findings of ducklings that not infected with virus or treated with planet extract showed normal histological structure of liver and spleen. (Fig 1.G1).

Group (2) (+ve control): Histopathological findings of ducklings that died after i/m inoculation with, $1x10^6$ EID_{50}/ML of isolated DHAV3 in liver and spleen. Liver showed severe hepatocytic degeneration with focal necrotic area and mononuclear cells infiltration. Spleen showed noticeable lymphocytic depletion with scattered lymphocytic necrosis. (Fig 1.G2).

Group (3) (Received Curcmin): The histopathological examination revealed less regenerated feature, liver showed diffuse degenerated hepatocytic cells with focal necrotic area and dilated hepatic sinusoids while the examined spleen revealed diffuse splenitis with lymphocytic depletion and lymphocytic necrosis. (Fig 1.G3).

Group (4) (Received Mint): The predominant histopathological lesions were represented by some regenerated feature with mild degeneration of hepatocytes and moderate splenitis and lymphocytic depletion. (Fig 1.G4).

Group (5) (Received Neem): The characteristic histopathological lesions were mild hepatitis with marked dilatation of hepatic sinusoids that infiltrated with lymphocytic cells while spleen showed mild lymphocytic depletion. (Fig 1.G5).

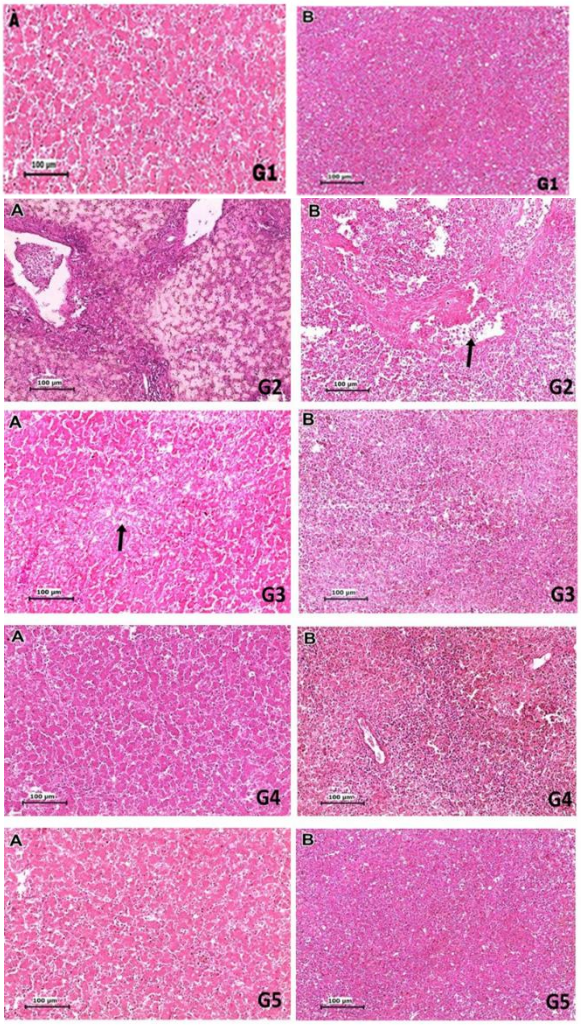

Figure 1 Photomicrograph showing the histopathological lesions of duckling tissues(**A** referred to liver, **B** referred to spleen) infected intramuscular with,$1x10^6EID_{50}$/ml of isolated DHAV3.G1(A,B)normal histological structure of liver and spleen.G2(A) hepatocytic degeneration and mononuclear cell infilteration.G2(B) lymphocytic depletion with scattered lymphocytic necrosis.G3(A) diffuse degenerated hepatocytic cells.G3(B) diffuse splenitis and lymphocytic necrosisG4(A)regenerated feature with mild degeneration of hepatocytes. G4 (B) moderate splenitis. G5 (A) mild hepatitis. G5 (B) mild lymphocytic depletion.

Table 2 Molecular screening for detection of DHAV3 using RT-PCR in different cloacal swabs at7th and 14th DPI.

No	DPI	Treatment	Result	CT	Titer (EID50/ml)
1		-	+	19.55	$5.566x10^4$
2		Curcmin	+	24.81	$1.511x10^3$
3	7th	Mint	+	30.29	$3.528x10^1$
4		Neem	+	27.32	$2.703x10^2$
5		-	+	17.12	$2.645x10^5$
6		Curcmin	+	22.15	$9.361x10^3$
7	14th	Mint	-	-	-
8		Neem	-	-	-

*CT means cycle threshold.

The results of the cloacal swap showed that all samples were positive for virus shedding by Real-time RT-PCR at the 7th day post infection.

The lowest virus titer was found in Ment treated birds (3.5 X 10 EID50/ml) followed by Neem treated birds (2.7 X 10^2 EID50/ml) then the Curcumin treated birds (1.5 $X10^3$ EID50/ml). The reduction in virus titer in comparison to

control untreated group (5.6 X 10^4 EID50/ml) was estimated as:

Curcumin 1 log reduction, Neem 2 log reduction and Ment 3 log reduction.

At the 14th day post infection, the virus shedding was positive only in Curcumin treated birds (9.3 X 10^3 EID50/ml) with nearly the same like in 7th day post infection and with 2 log reduction when compared to control untreated group (2.9 X 10^5 EID50/ml). However, virus shedding was stopped at 14th DPIMent and Neem treated group.

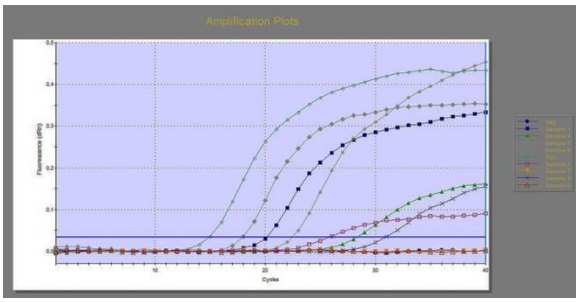

Figure 2 Molecular screening for detection of DHAV3 using RT-PCR.

Table (2) showed the Ct values of virus shedding by Real-time RT-PCR at the 7th and 14th days post infection (DPI) for the 3 treated groups (3, 4, 5) with Curcumin, Ment and Neem in comparison to control infected untreated group. Negative control (G1) has no Ct values, while Ct values of positive infected untreated control at 7th and 14th days post infection were 19.5 and 17 respectively. In the curcumin treated group Ct was24.8 at 7th and 22 at 14th DPI. The Ment and Neem treated groups showed Ct values of 30 and 27 at the 7th DPI respectively, while they were negative at 14th DPI. The results concluded that the Ment treated group showed the most effective virus Shedding reduction followed by Neem treated group then the curcumin treated group. The reduction in virus titer indicates possible antivirus effect of these herbal extracts on virus replication inside birds after infection.

4. DISCUSSION

DHAV is highly contagious viral disease causes severe economic losses among duck raising farms in Egypt and worldwide. In Egypt, several disease outbreaks in duckling farms were observed recently by Erfan et al. (2015) and El-Kholy et al. (2021).

In the present work, all investigated ducklings were manifestations including Imbalance, lethargy, and ataxia, falling on their sides and kick spasmodically followed by opisthotonos have been occurred in ducklings within 1-2 hours prior to death. The obtained results were like previously recorded by Zanaty et al. (2017) and Hassaan et al. (2018). The postmortem examination of recently dead and sacrificed ducklings showed mainly hemorrhagic liver. Similar result was reported previously by Hisham et al. (2020).

Results of virus isolation on ECE revealed that embryo deaths occurred after 5-7 days of inoculation in the first passage. The second passage showed embryo deaths after 3-5 days. These results are in accordance with (El kholy et al, 2021) who reported embryo died after 3-5 days post inoculation. The embryo mortalities were 56% in first passage and increased up to 84% in the second one. These results coincide with previous results of (Hassaan et al. 2018). The allantoic fluid was greenish. The collected embryos showed stunting, subcutaneous hemorrhage over the whole body with edema especially abdomen and hind limbs and necrotic liver. These findings go in parallel with *Hassan et al., 2020; Mansour et al., 2019and Yehia et al., 2020*. One of the most promising alternatives for controlling DVH is herbal products and botanicals. Our experimental evaluation of three natural substances (Curcumin, Mint and Neem) resulted in protection rate of 40%, 90% and 70% respectively. Curcumin resulted in a protection rate 40% and this result agreed with (Tamam et al. 2010) who reported that the chickens infected then treated with Curcumin showed protection (40%). Mint resulted in protection rate 90% and this result partially agreed with (Awaadet al.2016) who mentioned that mint has significant antiviral effect in chickens infected with Newcastle disease virus. Neem resulted in a protection rate of 70% and this result nearly similar with other result that reported by *Hegazy et al.2013*who found that neem provided 100% protection in ducks infected with avian influenza. The predominant histopathological lesions of ducklings that died after i/m inoculation with,1x10^6 EID_{50}/ML of isolated DHAV3 were found in liver and spleen. Liver showed severe hepatic degeneration with focal necrotic area and mononuclear cells infiltration. Spleen showed noticeable lymphocytic depletion with scattered lymphocytic necrosis. Those results agreed with that of other researchers Kamomae et al. (2017) and Zanaty et al. (2017) who mentioned that liver of affected ducklings with DHAV showed severe necrosis of hepatocyte associated with apoptotic bodies. The predominant histopathological lesions of the protected groups showed some regenerated feature of hepatocytes and moderate splenitis. Those results agreed with *(Hegazy et al.2013)* who reported the effect of herbal components as regenerative features of liver and spleen. Moreover, in case of mint and neem treatment groups virus shedding decreased (1log,3log and 2log) after 7days post infection and stopped shedding at the 14th day post infection in comparison to control untreated group.

These results agreed with those of other studies reported by Tamam et al. (2010) and Hegazy et al. (2013) who reported antiviral activity of herbal substances as an alternative solution.

5. CONCLUSION

In conclusion, the Ment extract was the most effective virus reduction followed by Neem than curcumin. The reduction in virus titer indicates possible antivirus effect of these plants on virus replication inside birds after infection.

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