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IN VITRO ANTIOXIDANT, HEMOLYTIC, THROMBOLYTIC POTENCIES OF *Centratherum anthelminticum* SEED EXTRACTSAND IT'S *IN OVO* ANTIVIRAL EFFICACY

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Herbal medicines have always been in practice since ancient times due to their ease and efficiency. Therefore, methanolic, acetonic, chloroform and n-hexane extract from the seeds of *Centratherum anthelminticum* were prepared by soxhlet extraction method. These extracts were subjected to quantify antioxidant abilities like TPC, TFC, DPPH free radical scavenging, reducing power, radical scavenging capacity for hydroxyl and nitric oxide. The toxicity of the extract was assessed by hemolytic assay *in vitro* on human and poultry erythrocyte. However, the percentage clot lysis behaviour of these extracts was also analyzed by thrombolytic activity by using Clopidogrel as standard. Further, Antiviral effect was inspected by *in ovo* analysis against Newcastle disease virus (NDV). Seven groups of nine-day-old embryonic chicken eggs were inoculated with different concentrations of extracts. Except for the negative control group; all groups were inoculated with NDV strain and different concentrations of four extracts. Observed the embryo's survival every day. Hemagglutination was done by collecting allantoic fluid from experimental eggs. Results indicated that all the extracts have antioxidant potential and display the highest antiradical behaviour in the pattern of methanolic > acetonic > chloroform > n-hexane. The hemolytic activity showed that all extracts of *C. anthelminticum* are non/less toxic towards Human and poultry RBCs (< 15%). The amount of clot lysis ranged from 19 to 51% compared to 65% and 6.4%, respectively provide by positive control and negative control. In the case of the methanol extract, the level of clot lysis was the most abundant (51%). In the case of *in ovo* antiviral assay, the highest survival rate was observed in the methanolic extract at 400µg/mL and acetonic extract at 300µg/mL as they control the NDV activity ultimately, evidenced by the absence of embryo death and HA titre. Chloroform and n-hexane could not inhibit the virus completely.

Keywords: Antioxidant, hemolytic, thrombolytic, Antiviral, Newcastle disease virus.

INTRODUCTION

Medicinal plants are used all over the world to treat various diseases, including inflammation, heart diseases, cancer, diabetes, aging, cardiac dysfunction and other degenerative disorders (Andleeb *et al.,* 2020). Phytochemicals have reported a significant impact to treat the various ailments, which suggests their therapeutic effect (Batool *et al.,* 2019). Diet rich in Phenolic and flavonoid possesses antioxidant activity, which has attracted the attention of scientists all over the world (Tungmunnithum *et al.,* 2018). Natural phenolic and flavonoid are secondary plant metabolites which possess hydroxyl group at aromatic rings (Lee *et al.,* 2015). Phenols are vital constituents of plants because of their scavenging ability due to their hydroxyl groups and may contribute directly to antioxidative action (Bendary *et al.,* 2013). Therapeutic plants and their related compounds can quench the free radicals by donating an electron, which eventually stops radical chain reaction, thereby protecting the body against different diseases (Narayanaswamy and Balakrishnan, 2011). They are well known as Metal chelators, Radical scavengers, reducing agents, contributors of hydrogen, and quenchers of singlet oxygen. Moreover, enzyme antioxidants may not be performed effectively under oxidative stress conditions and non-enzyme antioxidants are required to maintain adequate cellular functions (Kurutas, 2016). Antioxidants can shield cell components from oxidation damage and limit the risk of different oxidative stress-induced degenerative diseases (Merghem *et al.,* 2019).

In developing countries, synthetic antithrombolytic agents such as streptokinases (SC) or tissue plasminogen activators (t-PA) have increased popularity in the treatment of heart or thrombotic diseases due to their low cost (Kabir *et al.,* 2016). The related causes are unspecific medication activity, intracranial haemorrhage and extreme anaphylaxis. In this case, a growing number of researchers are using natural resources for evaluating safer and more effective antiplatelets, anticoagulants and antithrombotics (Talukder *et al.,* 2017).

Sensitivity to plant chemical elements is lively in the development of new pharmaceutical drug items. Plant extracts and natural products are an endless source of new antivirals (Ali *et al.,* 2019). There are currently limited, useful antiviral drugs to treat viral diseases. The unique herbal medicines need to be developed by current extraction techniques, biological processing and pharmacological efforts (Savithramma *et al.,* 2011). The identification of novel substances with intracellular and extracellular antiviral properties is the utmost requirement (Lin *et al.,* 2014).

There are many economically essential problems related to poultry, one of such is Newcastle disease (ND). Globally, poultry has undergone many socio-economic crises with each passing year due to this viral disease (Yune and Abdela, 2017). In Pakistan and India ND is generally called "Ranikhait" (Narayanan *et al.,* 2010). ND is the most severe threat irrespective of age and sex of poultry and other avian species (Iram *et al.,* 2014). Office International des Epizooties [OIE] reported it as LISTED disease due to its dreadful concerns (Boynukara *et al.,* 2013).

This drastic disease is caused byvirus Newcastle disease virus (NDV), is a member of the Paramyxoviridae family and exists as a variety of strains; velogenic, mesogenic and lentogenic (Ashraf *et al.,* 2017). NDV belonging to genotype VIId was associated with severe problems in oviducts, which leads to the production of soft-shelled, shell-less eggs and even decreased or loss of egg production (Li *et al.,* 2016).There have been several efforts including the use of live and killed vaccines, to control economic losses caused by this disease but no method imparted 100% immunity and hence proved ineffective (Raza *et al.,* 2015). Due to mutations in viral strains, NDVirus has become resistant and difficult to control, so it is important to look for alternative steps. In developing countries, plants are considered to be a preventive means in treatment for a variety of viral infections. Till the date, use of a combination of herbal medicine is considered as one of the efficient and less expensive ways to treat the virus as compared to other expensive means (Andleeb *et al.,* 2020).

Different strategies are needed to either prevent the replication of NDV or to decrease its drastic effects on an infected flock (Miller *et al.,* 2015). *Centratherum anthelminticum* belongs to Asteraceae family of flowering plants (which comprises more than 1000 species). It is commonly referred as kalijiri or bitter/black cumin. The seeds have a hot, harsh taste and the seed extract is frequently used as an astringent in traditional medicine, in skin diseases such as leukoderma, to treat ulcers as an anthelmintic (Hua *et al.,* 2012; Paydar *et al.,* 2013). Still, a hiatus is present to validate the plant's biological activity. This study focuses on phytochemicals, antioxidants and antiviral capacities to obtain data justifying the potential use of the selected plants in pharmaceutical as well as the poultry industry.

Hence the study aimed to assess the *in vitro* TPC, TFC, antioxidant, hemolytic, thrombolytic potencies and antiviral efficacy of four extracts of *C. anthelminticum* seeds against NDV *in ovo*.

MATERIALS ANDMETHODS

Plant materials and extraction procedures: The selected plant *C. anthelminticum* seeds were collected from Soan Skesar Valley, Punjab, Pakistan. Botanical identification was authenticated by the Department of Botany, GCUF. Twenty g aliquots were then extracted through soxhlet at a temperature not reaching the boiling point of the solvent by using methanol, acetone, chloroform and n-hexane. The extracts were filtered by a quantitative filter paper of Whatman ® and the residue was dry-coated at 40°C with a rotary evaporator. Up to the study, the extracts were lyophilized and preserved. The total yield of 20 g sample extraction was recorded in Table 2.

Phenolics and Flavonoids quantification: The plant phenolics (TPC) and flavonoids (TFC) were calculated using the methods adopted by Brighente *et al.* (2007). Final TPC content was expressed as (mg GAE/g) as gallic acid equivalents, while TFC result was expressed as (mg RU/g) as rutin equivalent.

In vitro Determination of antioxidant activities

***DPPH free radical scavenging assay*:** DPPH assay was evaluated for the free radical scavenging effect of *C. anthelmintic* seed extracts as defined by Sowndhararajan and Kang (2013) with slight modifications. Briefly, extracts concentrations (62.5, 125, 250, 500 and 1000 µg/mL) were added into freshly prepared DPPH solution (0.2 mM) and incubated in the dark at 27ºC for 30 min. After the incubation, the optical density was measured at 517 nm. A control experiment without the addition of plant extract was implemented. The DPPH radical scavenging activity (%) was calculated as per the following equation:

$$\text{Scavenging} = \frac{(\text{AC} - \text{AT})}{(\text{AC}) \times 100} \times 100$$

Where AC=Absorbance of the control, AT=Absorbance of the treated sample

***Determination of reducing power*:** For reducing power assay, different extracts concentrations (62.5, 125, 250, 500 and 1000 µg/mL) were mixed with freshly prepared phosphate buffer (0.2 M, pH 6.6) and 1% $K_3Fe(CN)_6$. The reaction mixture was heated at 50ºC for 20 min. After 20 min of incubation, TCA (10% w/v) was added and centrifuged (10000 rpm for 10 min). The resulting supernatant was then diluted with deionized water and freshly prepared $FeCl_3$ (0.1% w/v) solution. The optical density of the reaction mixture was measured at 700 nm (Sylvie *et al.,* 2014).

***Hydroxyl radical scavenging activity*:** The hydroxyl radical scavenging potential of *C. anthelminticum* seed extracts was determined by evaluating the degradation of deoxyribose into thiobarbituric acid reactive species (TBARS) as described by Bhat *et al.* (2018). The reaction mixture contained sodium

phosphate buffer (0.2 M, pH 7.0), 2-deoxyribose (10 mM), EDTA (10 mM), H_2O_2 (10 mM) and plant extract. The reaction mixture was incubated at 37ºC for 4 h. After incubation, TCA (2.8% w/v) and TBA (1% w/v in NaOH) were added to the reaction mixture and boiled for 20 min followed by cooling to room temperature. A control reaction mixture was also prepared without the addition of plant extract. The optical density of the reaction mixture was determined at 532 nm to determine the generation of TBARS. The hydroxyl radical scavenging efficacy was determined using the following equation:

$$\text{Scavenging} = \frac{(\text{AC} - \text{AT})}{(\text{ACl}} \times 100$$

Where AC=Absorbance of the control, AT=Absorbance of the treated sample

***Nitric oxide radical scavenging assay*:** The technique adopted by Ebrahimzadeh *et al.* (2008) was used to assess the scavenging ability of *C. anthelminticum* seed extracts against radical nitric oxide. Nitric oxide was produced from sodium nitroprusside and measured by the reaction of Greiss. Curcumin was taken as a standard. Curcumin prevents nitric oxide synthase activation and is a potent scavenger of nitric oxide that occurs naturally. This reduces the amount of nitrite created by sodium nitroprusside between oxygen and nitric oxide. The absorbance was estimated at 596 nm and the antioxidant activity percentage was determined using the equation formula.

$$\%\text{age inhibition} = \frac{(\text{Ao} - \text{A1})}{\text{Ao}} \text{X } 100$$

***Hemolytic activity*:** Procedure followed for hemolytic analysis was stated by Rubab*et al.* (2015) and Shabbir*et al.* (2015). Three mL of fresh blood from chicken and humans was collected separately. The supernatant plasma was discarded after centrifuged the blood samples for 5 min at 1000 x*g* plasma and washed the cells with 5 mL of chilled isotonic Phosphate-buffered saline (PBS) pH 7.4, three times. 100 µL of each extract was mixed with blood (10^8 cells/mL) individually. Samples were incubated at 37ºC for 30 min. Then, the samples were centrifuged for 5 min at 1000 x *g* immediately. The supernatant (100 µL) was taken from each tube and diluted 10 times with chilled PBS. PBS was taken as negative control and Triton X-100 as a positive control. The absorbance was intended at 576 nm using µQuant. The % age RBCs lysis for each sample was measured.

***Thrombolytic activity*:** Ali *et al.* (2014) defined the procedure to check the thrombolysis ability of plant extracts. Fresh human blood was collected in three different pre-weighed sterile microbes and allowed to incubate for 45 min at 37°C. After the formation of a clot, discharge the upper fluid from all micro-tubes. Distilled water and Clopidogrel was used as a negative and positive control. In each test tube, 100 µl of *C. anthelminticum* seed extracts are added and incubated for 90 min at 37°C. The liquid released from the clot was removed and the tubes were measured again to see the difference in weight when the lump was disrupted.

The following equation calculated the percentage of clot lysis:

$$(\%)\text{of clot lysis} = \frac{(\text{released clot weight})}{(\text{clot wt after clot disruption})} \text{x } 100$$

***In ovo antiviral assay*:** NDV strain (LaSota strain) was bought from the local market of poultry feed. Embryonated chicken eggs were incubated for 9 days in an incubator at 37ºC. Embryo Infectious Dose 50% of virus was recorded by Young *et al.* (2002).

Egg Inoculation: The embryonated eggs were inoculated with (methanolic, acetonic, chloroform and n-hexane**)** extracts and labelled them in groups according to the concentration of extract as shown in table 1. In all groups, Group 1 (G1) to G4 was inoculated with 0.2 mL of virus mixtures with at final concentration with 300, 400, 500 and 600 µg/mL for *C. anthelmsinticum* seeds methanolic, acetonic, chloroform and n-hexane extracts. G5 inoculated with the only 0.2 mL of virus and took as a positive control. G6 has eggs inoculated with extract only (no virus). In G7 all eggs were uninoculated (negative control). Inoculated sites were closed with paraffin then incubated at 37°C for 96 h. Tested eggs were perceived daily for the death of embryo till 72 h post-injection. After 96 h, cooled the eggs and embryos were observed for survival and growth. Further to perform for haemag glutination test, allantoic fluid from treated eggs was harvested to detect NDV.

***Hemagglutination test (HA)*:** To quantify the sum of the virus, HA titre test was used by using two-fold dilutions. The allantoic fluid was put by harvesting it from surviving embryos eggs. From harvested allantoic, 50 µL was serially diluted by 50 µL of normal saline in 96 well V bottom designed microtiter plate. Then, to each well 50 µL of RBCs 1% (freshly collected from chicken) was added and mixed gently. Allow them to stand for 25 minutes at room temperature after sometime virus titre was noted as reciprocal of the highest dilution that triggered agglutination of chicken RBCs (Khaldoun, 2016).

Table 1. Grouping of embryonated eggs inoculated with different concentrations of *C. anthelminticum* extracts.

Group (G) n=7	Treatment
G1	Methanol/Acetone/chloroform/n-hexane seed extract of *C. anthelminticum*300 µg/mL + 0.2mL 4HA Virus
G2	Methanol/Acetone/chloroform/n-hexane seed extract of *C. anthelminticum*400 µg/mL + 0.2mL 4HA Virus
G3	Methanol/Acetone/chloroform/n-hexane seed extract of *C. anthelminticum*500 µg/mL + 0.2mL 4HA Virus
G4	Methanol/Acetone/chloroform/n-hexane seed extract of *C. anthelminticum*600 µg/mL + 0.2mL 4HA Virus
G5	Virus control
G6	Extract control
G7	Untreated embryonated chicken egg

Statistical analysis: All experiments were conducted as triplicate and the data were reported as mean ± standard deviation (SD). The HA titre were presented by square root of standardize values.

RESULTS

***Phenolics and Flavonoids quantification*:** The amounts of total phenolics and flavonoids were detected in the methanolic, acetonic, chloroform and n-hexane seed extracts of *C. anthelminticum* and the results are summarized in Table 2.

Table 2. Total yield, TPC and TFC of *C. anthelminticum* seed extracts.

C. anthelminticum	Total yield (g)	TPC (mg GAE/g)	TFC(mg RU/g)
Methanolic extract	2.7	19.50±0.50	15.30±0.12
Acetonic extract	2.1	16.35±0.35	9.67±0.33
Chloroform extract	1.6	10.10±0.20	6.56±0.23
n-hexane extract	1.1	6.40±0.45	2.76±0.55

***DPPH radical scavenging method*:** Result of antioxidant activity rationalized by DPPH indicated that the extracts showed the concentration-dependent response, the scavenging power increase as the concentration of extract increase. Highest radical scavenging was recorded in methanol > acetone > chloroform > n-hexane extract of *C. anthelminticum* shown in figure 1(A).

***Reducing power activity*:** The increasing reducing power corresponds to the increase in the optical density. Results of this activity showed that extracts of *C. anthelminticum* exhibited good reducing power with increasing concentrations from 62.5 to 1000 µg/mL. Methanolic extract showed significantly higher reducing power as followed by acetone > n- hexane > chloroform ($p<0.05$) Figure 1(B).

***Hydroxyl radical scavenging activity*:** The analysis of OH scavenging activity also revealed the maximum scavenging activity was achieved by methanolic extract followed by acetone > chloroform > n-hexane indicated in Figure 1(C).

***NO radical scavenging assay*:** The findings of the nitric oxide scavenging test were not as good as the other assays; extracts showed moderate nitric oxide activity, Fig. 1(D). Highest

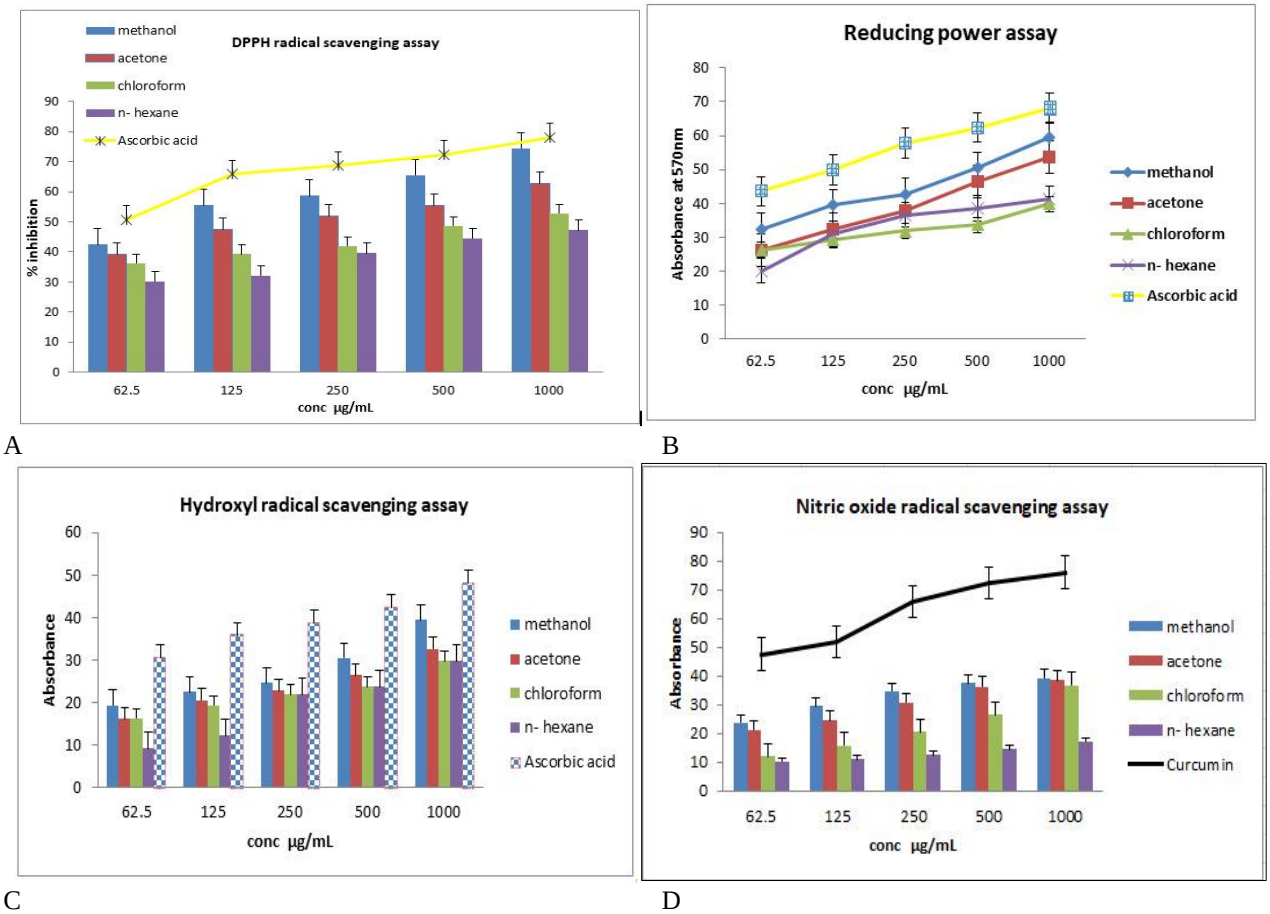

Figure 1. *In vitro* antioxidant activities of *C. anthelminticum* seed extracts. A: DPPH free radical scavenging activity; B: Reducing power; C: Hydroxyl radical scavenging activity; D: Nitric oxide antioxidant activity.

activity was showed by methanolic extract at 1000 µg/mL (39.4±0.05) and the least activity was showed by n-hexane i.e., (17.24±0.03).

Hemolytic assay: Hemolytic potential of *C. anthelminticum* seeds extracts was expressed in percentage hemolysis and reported as mean ± standard deviation of three replicates shown in (Table 3). All extracts showed minimal hemolytic effects on human and chicken erythrocytes. Such findings demonstrated that if medicinal formulations from this plant, seeds are used at low concentration, parameters of the RBC membrane are not changed.

Table 3. Percentage IC_{50} of hemolytic activity of different concentrations of *C. anthelminticum* extracts.

Sample name	Human Blood	Chicken Blood
Methanolic extract	1.09±0.45	0.70±0.45
Acetonic extract	1.93±0.15	1.03±0.15
Chloroform extract	2.32±0.70	1.62±0.70
n-hexane extract	2.74±0.30	1.93±0.41
PBS	0.63±0.19	0.97±0.51
Triton-X	96.58±1.72	94.55±1.55

***Percentage thrombolysis*:** The percentage clot lysis of different extracts of *C. anthelminticum* seedsand controls is presented in Fig. 2. The application of positive and negative control to the clots, namely Clopidogrel and distilled water, showed 66 and 6.4% respectively of clot lysis activity. In contrast, the percentage of clot lysis accounted for *C. anthelminticum* extracts 51, 47, 32 and 19% for methanolic, acetonic, chloroform and n-heaxane extracts, respectively. Methanolic, acetone extracts showed significant clot lysis in contrast to positive and negative standards.

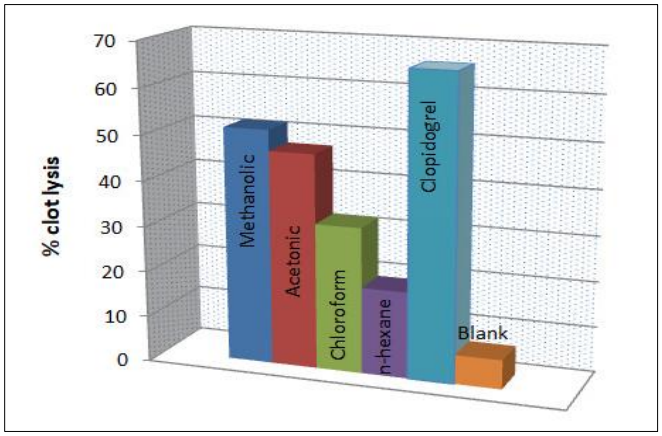

Figure 2. Percentage clot lysis of *C. anthelminticum* seed extracts with positive and negative controls.

***Median percent Embryo Infectious Dose*:** Applying the index formula to the dilution immediately resulted in the infection rate above 50 percent=$10^{-3.5}$. This virus suspension dilution contained one virus EID50 unit in 0.2 mL. 1mL of the suspension virus should contain 10 times the measured dilution reciprocity.

$$EID_{50}/mL = 10 \times 10^{-3.5} = 10^{-4.5}\ EID_{50}/mL.$$

***In ovo antiviral activity of different extracts C. anthelminticum*:** In this study, all chicken embryos died within 48 h post-inoculation with NDV, it was a strong warning that the virus strain is virulent to eggs. However, the addition of any four tested extracts of *C. anthelminticum* significantly prolonged the time of survival of embryos in a dose-dependent manner. In methanolic extract-treated group concentration of 400 µg/mL had complete inhibition of NDV replication with 0% mortality (Table 4). Haemag glutination showed 0 HA titre it means that this concentration controlled virus from the beginning. 300 and 500 µg/mL has 20% mean mortalities at 48 h and has no mortality till 72 h. 300 µg/mL has 4 HA titre; it controls the virus replication earlier than 500µg/mL, which has haemag- glutination at 8. The highest concentration (600 µg/mL) cause 40% mortality after 72 h post-inoculation with HA titre of 32 (table 5). It may suggest that all the concentrations have the ability to inhibit virus multiplication.

Table 4. Embryo deaths following inoculation of embryonated chicken eggs with Newcastle disease virus (NDV) and different concentrations of different extracts of *C. anthelminticum*.

Treatment	Concen-tration µg/mL	Mortality with different time intervals			% Mortality
		24 h	48 h	72 h	
Methanolic Extract	300 µg/mL	0	1	0	20%
	400 µg/mL	0	0	0	0%
	500 µg/mL	0	1	0	20%
	600 µg/mL	0	1	1	40%
Acetonic Extract	300 µg/mL	0	0	0	0%
	400 µg/mL	0	1	0	20%
	500 µg/mL	0	0	1	20%
	600 µg/mL	0	1	2	60%
Chloroform Extract	300 µg/mL	0	1	1	40%
	400 µg/mL	0	0	1	20%
	500 µg/mL	0	1	1	40%
	600 µg/mL	1	1	1	60%
n-hexane Extract	300 µg/mL	1	1	1	60%
	400 µg/mL	1	2	0	60%
	500 µg/mL	0	1	1	40%
	600 µg/mL	1	1	0	40%
VC	-	2	3	-	100%
EC	-	0% mortality.			0%
Uninoculated Group	-	0% mortality.			0%

In acetonic seeds extract-treated group eggs, 300 µg/mL inoculated group showed 0% mortality throughout three days (72 h) post-inoculation showing HA titres of 0. 400 and 500 µg/mL inoculated groups have 20% mortality indicating that both have the same capability to control the virus. The high death rate was observed in 600 µg/mL treated group and

showing the HA titre is 128. It can be interpreted that this dose has a lethal effect itself on embryo survival (table 4 & 5). High mortality was shown by 600 µg/mL of different extract at 24, 48 and 72 h time intervals.

The three days record of chloroform seeds extract of *C. anthelminticum* inoculated group indicated that there is no complete restriction of antiviral potential was noticed using different concentrations of plant extract. There is no mortality at 24 h in first three concentrations. After 48 h there is 1 embryo died in both 300 and 500 µg/mL. Total mortality is 40% till 72 h in both groups. HA titre showed different titre values regarding their aptitude to control the viral effects. 600 µg/mL has 60% mortality 72 h post-inoculation. From the results, it may be concluded that the dichloromethane extract of this plant has less ability to control virus propagation.

N-hexane extract of *C. anthelminticum* displayed the dose-dependent response by the level of virus control. In this extract 300 and 400 µg/mL have shown 60% mortality (table 4). In HA titre 1024 of 300 µg/mL indicates that this concentration of PE did not control virus replication while 400 µg/mL group showed HA titre 512. 500 and 600 µg/L has fewer mortality rates than first two concentrations, i.e. 40%. High concentration 500 µg/mL showed 20%, 250 µg/mL showed 40% and low concentration of *Ceratonia siliqua* caused 80% mortality.

Table 5. Mean hemagglutination (HA) titres in embryonated chicken eggs inoculation with NDV and different concentrations of different extracts of *C. anthelminticum.*

Treatment	Concentration µg/mL	HA titre
Methanolic Extract	300 µg/mL	4
	400 µg/mL	0
	500 µg/mL	8
	600 µg/mL	32
Acetonic Extract	300 µg/mL	0
	400 µg/mL	4
	500 µg/mL	8
	600 µg/mL	128
Chloroform Extract	300 µg/mL	32
	400 µg/mL	8
	500 µg/mL	128
	600 µg/mL	512
n-hexane Extract	300 µg/mL	1024
	400 µg/mL	512
	500 µg/mL	512
	600 µg/mL	128
Virus Control	-	2048
Extract Control	-	0
Uninoculated Eggs	-	0

DISCUSSION

Plant therapy is a result of their phytochemical prosperity (Jimoh *et al.,* 2019). The nature of plant-based phytochemicals influences its antioxidant function. Such phytochemicals include phenolic flavonoids and alkaloids that assume covering jobs in plant protection components, single-point oxygen foragers, allelopathy, high-vitality radiation safeguards, decrease operators, pollinator fascination (Nascimento *et al.,* 2018). Due to their cell stabilization properties, phenolics have remedial capacity against various types of illness. Flavonoids are a series of polyphenolics in many plants which are responsible for different biochemical function (Gul *et al.,* 2013).

In this study, seed extracts of *C. anthelminticum* were prepared by using methanol, acetone, chloroform and n-hexane. All of these extracts were tested for their biological potentials by various *in vitro*, for antioxidant potencies and also for their hemolytic and thrombolytic activities. Among the different techniques used to calculate the antioxidant strength, DPPH (2,2-diphenyl- 1- picrylhydrazyl) is the easiest, simple and most budget-effective process. All extracts of *C. anthelminticum* were able to decolourize purple-coloured DPPH radical to yellow colour (shown in Fig. 1). Other antioxidant parameter helps to assess the reduction power and has been associated with plant phenolics by redox reactions (Talukder *et al.,* 2017). Due to the presence of antioxidant properties in the *C. anthelminticum* seed extracts, an oxidized form of iron (Fe_3^+) could be reduced to its reduced form (Fe_2^+). From the result (Fig. 1B), it is predicted that the antioxidants present in the extract helped reduce the ferrous structure of the ferricyanide complex (Fe_3^+).

Hydroxylic radicals are very reactive and of short duration (Hayyan *et al.,* 2016). They can cause destructive effects on major macromolecules, including nucleic acids and proteins. Similarly, many plants extracts and flavonoids, including mangiferin and naringin, have been found to scavenge OH free radicals in a concentration-dependent manner (Markovi *et al.,* 2017). Many flavonoids synthesized as secondary metabolites by different plants were previously reported to scavenge OH radicals (Lushchak, 2014). Nitric oxide is an essential cell stimulating agent implicated in specific biochemical and pathological processes. It is a potent vasodilator with a brief half-life in the blood for a few seconds (Duarte *et al.,* 2014). After reaction with oxygen or superoxide radicals, the nitric oxide radical is toxic. Various extracts of *C. anthelminticum* seeds reduced concentration-dependent production. In a concentration-dependent manner, several plant extracts and plant formulations were recorded to scavenge nitric radicals (Fig. 1D).

Toxicity is an essential factor in the design of pharmaceutical medicines and an important starting point for hemolytic actions, providing superior knowledge on the interaction at the cellular level between molecules and biological entities.

Hemolytic activity of any compound is an indication of general cytotoxicity towards normal healthy cells (Da Silva *et al.,* 2004). Table 3 showed that all four extracts of *C. anthelminticum* showed low hemolytic effects towards human and chicken RBCs which can say that if *C. anthelminticum* use in medicine will not cause toxic effects. In the study of thrombolytic activity, the percentage of positive and negative regulation of the clot lysis shows clearly that water could not affect clot dissolution. Besides, the controls (positive and negative) and different extracts of *C. anthelminticum* seeds were exposed to significant differences in the value of clot lysis.

The underlying mechanism of plant extracts to control NDV *in ovo* replication is not known yet. This exposes the inhibitory rather than virucidal potential of the extract on this virus at these doses. However, many traditional medicinal plants that are used to cure viral diseases have been exposed to contain different types of substances. Notable examples of these metabolites comprise of; alkaloids, coumarins, terpenes, flavonoids, anthraquinones and naphthoquinones (Sulaiman *et al.,* 2011). These compounds exert their effects by killing the virus and interfering with viral replication (Andleeb *et al.,* 2020). Acetonic extract and methanolic extract of *C. anthelminticum* at 300 µg/mL and 400 µg/mL exhibited the complete inhibition of NDV without prompting mortality of any chicken embryo. These findings are in line with results of Ashraf *et al.* (2017) who documented that low concentrations 300 µg/mL of methanolic and 400 µg/mL ethanolic extract of *Glycyrrhiza glabra* produce 0% mortality. The response of virally infected embryo is different depending on extract type and concentration supplied. Some extracts of plant expressed a dose-dependent relationship with the virus while others cause toxicity for embryos. The positive responses provided by extract are believed to be as an effect of phytochemicals present in that extract or combination with other compounds.

***Conclusion*:** It's evident from the above findings that, the *C. anthelminticum* seed extracts have antioxidant and thrombolytic activity and their strength varies in different extracts. Evidence from hemolytic assay showed that the natural resources of this plant extract are taken into account for the health of both humans and animals. This is a preliminary study and it is necessary to assure the nontoxic nature and dose-activity relationships of *C. anthelminticum.* The antiviral test indicates that the methanol and acetone extracts have significant antiviral capacity in experimental local chicken*s in ovo* against Newcastle disease infection. Administering this extract can helptoreduce the implications of NDV infection a healthier approach.

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