



In Vitro Evaluation of Antiviral Potential of Some Plants Water Extract Against Bovine Coronavirus



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CORONAVIRUS, particularly the genus Betacoronavirus, has been identified as a deadly source of numerous acute respiratory epidemics worldwide, affecting both humans and animals. The genus includes Covid-19, Severe acute respiratory syndrome coronavirus, and Middle East respiratory syndrome-related coronaviruses. In contrast, others are expected to have the risk of transformation such as bovine and porcine coronavirus. We screened a set of natural plant water extract which are red onion (*Allium cepa*), shallot (*Allium ampeloprasum*), watercress (*Eruca sativa*), and wormwood (*Artemisia absinthium*) that weighed and dissolved into a specific concentration then filtrated by specific biological filters. These extracts are known for their strong inhibition of the cytopathogenic effect of many pathogens. The cytotoxicity assay of the extracted plants was evaluated by the MTT assay using serial concentrations from the red onion and shallot extracts (10-100 ug) and wormwood and watercress extracts (20-200ug). The antiviral effect was evaluated using the concentrations mentioned above of the plants water extracts by application on the Madin Derby Bovine Kidney (MDBK) cell line infected with the virus. It showed pronounced inhibition of the bovine coronavirus (BCoV) in comparison to the control untreated cell line by both microscopic examination, and optical density index measurement. This work encourages the application of these extracts in trials of the treatment of the clinical cases of infected animals with coronavirus infection. It has the potential as a novel approach against coronavirus infections in animals.

Keywords: *In vitro*, Plants, Bovine coronavirus, Antiviral, Water extract.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which cause worldwide Covid-19 pandemic infection, becomes the major worldwide important crisis facing human recently.

A new antigenically related variant was recorded due to the circulation of virus from human to animal and vice versa as in (mink animal) which indicate unexpected future mutations and different virus genetic changes [1]. These huge and rapid variations lead the world to the idea

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that the vaccination is not enough to face future attacks where the viral mutations do not give enough protection with limited specifications. In addition, it depends on immunity at the host side either human or animal vaccination success. So, the world critically need sa safe and natural way of treatment with direct interference effect on viral life cycle [2].

Other coronaviruses circulate in animal blood especially the ruminants close in genetic and pathogenesis profiles to the Middle East respiratory syndrome-related coronavirus (MERS) which is transmissible between humans and between human and camels [1]. Many plants naturally growing in the Egyptian environment were recorded to have antimicrobial activities beside the antioxidant and anticancer effect. Some of the most popular plants are red onion (*Allium cepa*), shallot (*Allium ampeloprasum*), watercress (*Eruca sativa*), and wormwood (*Artemisiaabsinthium*). The antimicrobial activities may be due to their active ingredients such as flavonoids, organosulfur compounds and alkaloid salts [3, 4]. In the current study, the antiviral effect of the water extract of the mentioned plants was evaluated *in-vitro* in the Madin-Darby bovine kidney (MDBK) cell line using the bovine coronavirus as a model of the genus Betacoronavirus.

Material and Methods

Virus

Bovine coronavirus (BCoV) (Mabus strain) 10^6 TCID₅₀/ml were kindly received from the Veterinary serum and vaccine research institute (VSVRI), Ministry of Agriculture, Egypt. The reference strain was used in the evaluation of the plants antiviral effect and safety process.

Cell line

Madin Derby Bovine Kidney (MDBK) cell line was supplied from VSVRI for *in-vitro* evaluation of the plant extract.

Preparation of plants water extract

Cold extraction [5]

The extraction was carried out by repeated cycle of freezing and thawing of green leaves of red onion (*Allium cepa*), shallot (*Allium ampeloprasum*), watercress (*Eruca sativa*), and wormwood (*Artemisiaabsinthium*). Then mixing and sieving process were applied. The final water extract is dried in the microwave oven till obtaining the dried powder .

Microwave-assisted extraction (MAE) [6]

By adding double-distilled water (DDW) to the dried plants by ratio 2:8 then left for cooling, sieving and then dried by microwave oven.

In-vitro studies

Infectivity test of BCoV to measure the titre of virus suspension

It was carried out according to Yesilbag et al. [7] with modifications. MDBK cells were seeded in growth media in a 96-well flat-bottom cell culture plate (150µl/well) then incubated at 37°C for 24-48 hours. When the cell line appeared confluent, the growth media were decanted. Tenfold virus (10^6 TCID₅₀/ml) serial dilution of the stock virus was prepared and then cells were infected with 100µl of the diluted virus. Maintenance media were added and the plates were kept in an incubator under daily observation. Cytopathic effects were recorded daily for 3-7 day. The 50% tissue culture infective dose (TCID₅₀) per ml was calculated according to Reed and Muench, [8].

Cytotoxicity assay of prepared dissolved plant extract by MTT assay

It was carried out on MDBK cell line simulated the method [9]. The wells with and without cells were incubated with 1 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 2hrs as previously described. MTT solution was discarded and 100 µL dimethyl sulfoxide (DMSO) was added per well. Formazan formation was quantified via photometric evaluation of the absorbance at 550 nm using the Synergy HTX Multi-Mode Reader (BioTek, Winooski, VT, USA).

Statistical analyses

Statistical analysis was performed with the aid of Microsoft Excel 2010 (Microsoft Corp., USA). Data were compared using the unpaired Student t-test. A p-value <0.05 was considered statistically significant.

Results

The table clarifies the dilutions used to measure the end point for bovine coronavirus used in the experiment. 10^7 /ml of viral suspension was used.

TABLE 1. The titration infectivity measure of BCoV in micro culture plate seeded by tenfold serial dilution.

Microplate wells	Virus dilutions										Control
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	
A	Cytopathic effect							Healthy normal cells			
B	Cytopathic effect							Healthy normal cells			
C	Cytopathic effect							Healthy normal cells			
D	Cytopathic effect							Healthy normal cells			
E	Cytopathic effect						Healthy normal cells	Healthy normal cells			
F	Cytopathic effect					Healthy normal cells		Healthy normal cells			
G	Cytopathic effect				Healthy normal cells			Healthy normal cells			
H	Cytopathic effect			Healthy normal cells				Healthy normal cells			

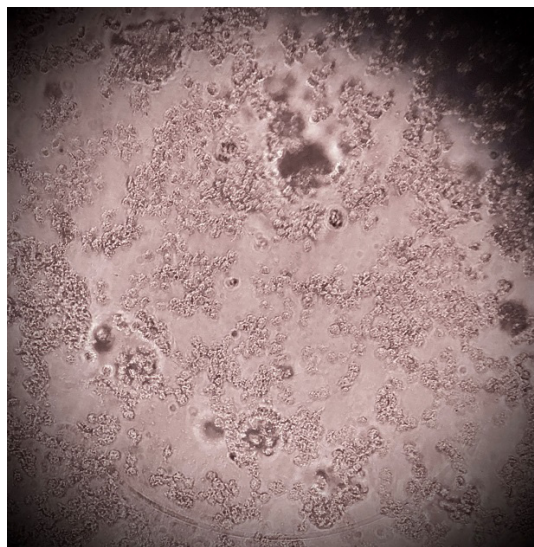


Photo (1a) MDBK cells inoculated with a combination of BCoV and plant extracts showing no cytopathic effect (CPE).

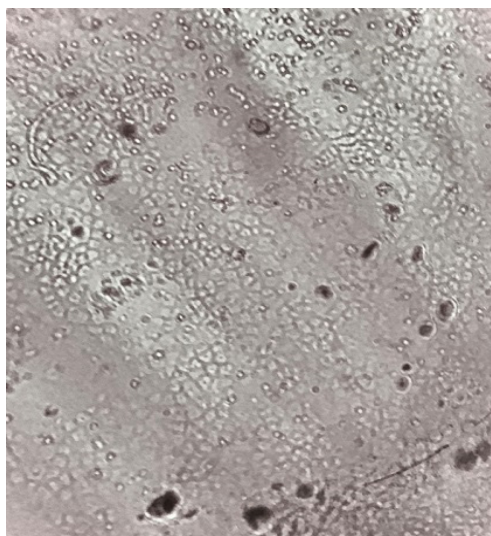


Photo (1b) MDBK cell infected by BCoV, CPE was clear as detaching of the cells with giant cell appearance.

The table represents the viability of cells in response to plants extract concentrations of red onion and shallot, demonstrating that the cytotoxic dose for both was 100µg dried extract/well.

The table shows how varied extract concentrations of wormwood and watercress affected cell viability, revealing that the cytotoxic dose for both was 200 µg dried extract /well.

The table shows the variable viability reactions of MDBK cells when exposed to

various plant extract concentrations of red onion and shallot, as measured by optical density from an ELISA reader in different sample wells. The minimal effective dose to inhibit viral invasion was 20 and 10 µg dried extract / well.

The table illustrates the variable viability reactions of MDBK cells when exposed to various plant extract concentrations of wormwood and watercress, as measured by optical density from an ELISA reader in different sample wells. The lowest effective dose to prevent viral invasion was 20 µg dried extract / well.

TABLE 2. Cytotoxicity assay of prepared water extract of Red onion and shallot (ug) inoculated in MDBK cell line by MTT assay expressed in mean OD index and calculated at wavelength 590.

	Red onion and shallot concentrations (µg)									
	10	20	30	40	50	60	70	80	90	100
Red onion	0.097	0.095	0.095	0.094	0.094	0.094	0.093	0.092	0.092	0.065
Shallot	0.097	0.097	0.097	0.097	0.095	0.095	0.094	0.093	0.093	0.059

TABLE 3. MTT cytotoxicity assay of prepared water extracts of wormwood and watercress (µg), inoculated in MDBK cell line, expressed as mean OD index, and estimated at wavelength 590.

	Wormwood and Watercress concentrations (µg)									
	20	40	60	80	100	120	140	160	180	200
Wormwood	0.098	0.098	0.098	0.097	0.097	0.097	0.097	0.097	0.096	0.055
Water cress	0.096	0.096	0.096	0.095	0.095	0.095	0.094	0.094	0.094	0.052

TABLE 4. *In-vitro* antiviral assay of red onion and shallot water extract (µg) inoculated in MDBK cell line using MTT assay with mean OD index determined at wavelength 590.

	Red onion and Shallot concentrations (µg)									
	10	20	30	40	50	60	70	80	90	
Red onion	0.064	0.095	0.095	0.094	0.094	0.092	0.092	0.092	0.092	
Shallot	0.096	0.095	0.095	0.094	0.094	0.094	0.092	0.092	0.092	

TABLE 5. *In-vitro* antiviral assay of water extract of wormwood and watercress (μg) by MTT assay expressed in mean OD index measured at 590 wave-length, inoculated in MDBK cell line.

	Wormwood and Watercress concentrations (μg)								
	20	40	60	80	100	120	140	160	180
Wormwood	0.096	0.096	0.095	0.095	0.095	0.095	0.095	0.093	0.092

Discussion

In the past decades, several researches around the world have been detected huge potentials of plants extracts. Plant extracts medicines and their activity is attributed to their multitude of constituents in its extracts. They are generally broken up into primary and secondary groups according to the purposes of products extracted [10]. Many potential nutritional and therapeutic extracts that apply to the antimicrobial scope have been recorded by many researches around the world, particularly secondary metabolites such as flavonoid and organosulphur compounds, which are not required for the day-to-day functions of plant cells [11].

Hence, many researchers have already used the antiviral approach in combination with different antimicrobial agents to face various infections in animals, but it still had limited effects in most clinical cases either due to toxic effective dose or expected dangerous side effects or low efficacy [12].

The maximum harmful dose for red onion and shallot discovered by OD parameter on MDBK cell line in comparison to control untreated normal cell parameter was ($90\mu\text{g}/\text{well}$) ($P=0.9$) of dried water extract, according to the data in Table 2. In case of watercress and wormwood the maximal safe dose was ($180\mu\text{g}/\text{well}$) ($P=0.7$) in Table (3) that showed safety on the MDBK cells. Regarding the antiviral effects of the Egyptian plants water extract on BCoV, the antimicrobial agent theory agrees with [13].

In comparison to the uninfected cells control, the lowest dose of extract that satisfied the mean OD parameter was directed to red onion (*Allium cepa*), shallot (*Allium ampeloprasum*), watercress (*Eruca sativa*), and wormwood (*Artemisiaabsinthium*) (20,10,20, and $20\mu\text{g}/\text{well}$, respectively) as shown in Tables 4 and 5.

The red onion and shallots used on the MDBK cells had no effect on the cell lines ($P=0.2$). In contrast, there was a significant difference ($P=0.04$) between wormwood and watercress concentrations applied on BCoV-infected MDBK cells. The results are in harmony with that obtained previously [14]. Where they proved the antiviral effect of the artesunate, a variant constructed from Artemisia, on the bovine herpesvirus-1 (BoHV-1) *in-vitro*.

Before cell inoculation, the virus was incubated with the extracts to allow the interactions. Except for *Allium ampeloprasum*, which was efficacious at the pre-infection level, all extractions were found to be efficient at both levels of inoculation. This suggests that the used plants extract in this study have inhibitory effect on viral multiplication, which is consistent with this theory of the mode of action [13].

After using a dose equivalent basis calculation, this result will be used to create a clinical trial approach in animals [15].

Conclusion

The water extract of (*Allium cepa*, *Allium ampeloprasum*, *Eruca Sativa* and *Artemisiaabsinthium*) proved to be effective antivirals against bovine coronavirus infection *in-vitro* with a high safety range. This result will be considered a milestone of clinical trial approach in the animals after applying specific dose equivalent basis calculation.

Conflict of interest

The authors declare no conflict of interest.

Authors' contribution

All authors contributed to the design and implementation of the research, analysis of the results and to the writing of the manuscript.

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تقييم النشاط المضاد للفيروسات لبعض خلاصة النباتات ضد فيروس كورونا معمليا

مها رأفت عبدالفضيل^١، احمد محمد علام^{٢*}، صلاح جليظ^٣ و محمد كرم البيومي^٤

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أصبح فيروس كورونا 2 (SARS-CoV-2) الذي يسبب عدوى جانحة Covid-19 في جميع أنحاء العالم ، هو الأزمة العالمية الكبرى التي يواجهها الإنسان مؤخرًا. تم تسجيل متغير جديد مرتبط بالمستضدات بسبب دوران الفيروس من الإنسان إلى الحيوان والعكس كما في (حيوان المنك) والذي يشير إلى طفرات مستقبلية غير متوقعة وتغيرات جينية مختلفة للفيروس. هذه الاختلافات الهائلة والسريعة تقود العالم إلى فكرة أن التطعيم لا يكفي لمواجهة الهجمات المستقبلية حيث لا توفر الطفرات الفيروسية حماية كافية بمواصفات محدودة. بالإضافة إلى ذلك ، فإنه يعتمد على المناعة في الجانب المضيف سواء نجاح التطعيم البشري أو الحيواني. لذلك ، يحتاج العالم بشكل حاسم إلى طريقة آمنة وطبيعية للعلاج مع تأثير التداخل المباشر على دورة حياة الفيروس.

تم تسجيل العديد من النباتات التي تنمو بشكل طبيعي في البيئة المصرية بأن لها أنشطة مضادة للميكروبات بجانب تأثير مضادات الأكسدة والمضادة للسرطان. من أشهر النباتات البصل الأحمر (*Allium cepa*) والكراث (*Allium ampeloprasum*) والجرجير (*Eruca sativa*) والأفسنتين (*Artemisia absinthium*). قد تكون الأنشطة المضادة للميكروبات ناتجة عن مكوناتها النشطة مثل مركبات الفلافونويد ومركبات الكبريت العضوي والأملاح القلوية. تُعرف هذه النباتات بتثبيتها القوي للتأثير الممرض للخلايا للعديد من مسببات الأمراض. في الدراسة الحالية ، تم تقييم التأثير المضاد للفيروسات لمستخلص الماء من النباتات المذكورة في المختبر في خط خلايا (MDBK) باستخدام فيروس كورونا البقري كنموذج لجنس فيروسات بيتاكورون.

قمنا بفحص مجموعة من مستخلصات المياه لهذه النباتات الطبيعية. حيث وزنت و أذيتت بتركيزات معينه من ثم تم ترشيحها بواسطة مرشحات بيولوجية محددة .. تم تقييم السمية الخلوية للنباتات المستخرجة بواسطة مقايسة MTT باستخدام تركيزات متسلسلة من مستخلصات البصل الأحمر والكراث (١٠-١٠٠ ميكروغرام) ومستخلصات الشيح والجرجير (٢٠-٢٠٠ ميكروغرام). تم تقييم التأثير المضاد للفيروسات باستخدام التراكيز المذكورة أعلاه لمستخلصات ماء النبات عن طريق التطبيق على خط الخلايا المعملية المصابه بالفيروس. أظهرت المستخلصات تثبيطًا واضحًا لفيروس كورونا البقري (BCoV) مقارنةً بخط الخلية غير المعالجة المتحكم فيه من خلال الفحص المجهرى.

أثبت المستخلص المائي للنباتات المستخدمه فعاليته في مكافحة عدوى فيروس كورونا البقري في المختبر مع نطاق أمان عالي. يشجع هذا العمل على تطبيق هذه المستخلصات في تجارب علاج الحالات السريرية للحيوانات المصابة بعدوى فيروس كورونا مع إعتماده كنهج جديد ضد عدوى فيروس كورونا في الحيوانات.