In vitro antiviral potential of *Ocimum basilicum* and *Olea europaea* leaves extract against Newcastle Disease Virus of poultry

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Summary

This study was carried out to investigate the effect of using alcoholic *Ocimum basilicum* and *Olea europaea* leaves extract, to study the antiviral activity against the Newcastle disease virus of poultry chicken embryo fibroblast monolayer culture. The extract was also decided for the chicken fibroblast culture and the concentrations (500, 250, 125, 100 and 50 µg /ml) of an alcoholic extract of *Ocimum basilicum* and *Olea europaea* leaves in maintenance media appeared to be nontoxic. Where the cytopathic effects of the Newcastle disease virus on chicken embryo fibroblast monolayer culture are well established so these concentrations were used to detect the antiviral activity of *Ocimum basilicum* and *Olea europaea* in addition to *in vitro*, pre and post -inoculative treatment of fibroblast cells with plant extracts to get an idea of viral reduction titer in cell tissue culture. Absence of cytopathic effects in monolayer cell culture and virus titer reduction were used as an indicative of antiviral activity of extract of *Ocimum basilicum* and *Olea europaea* leaves. *In vitro*, pre and post -inoculative treatment of fibroblast cells with plant extracts showed that leaves of *Ocimum basilicum*, and *Olea europaea* had antiviral activities of Newcastle disease virus at 500 and 250 µg/ml. In conclusion the viral reduction titer was found range between 10^{-6} to 10^{-1} with 500 µg /ml of *Olea europaea* and *Ocimum basilicum* extract as compared to 10^{-7} with virus control.
Keywords: *Ocimum basilicum, Olea europaea, Plant extracts,* Antiviral activity, New Castle Virus.

Introduction

Over the centuries, several important traditional medicinal systems such as Greek, Chinese, Tibetian, Indian, Siddha and Mediterranean have been evolved and established all over the world with the use of natural present active principles of plants in the form of various preparations for the relief of human and veterinary diseases (1). Recently appeared in a great desire in the use of medicinal plants in the treatment of many pathogens, to mostly devoid of side effects and ease of handling, and they contain a lot of active ingredients, plants have long been a very important source of new drugs, many plant species have been screened for substances with prophylactic activity, for that medicinal plants are a promising source of anti-viral activity (2). These plants are not used as whole rather their specific parts are recommended for the medicinal values in the traditional system (1 and 3). Leaf of the most important medicinal plants are *Ocimum basilicum* and *Olea europaea*, these plants are an important medicinal plant as they are a well-known source of different phytochemicals, they are distributed throughout most of the world and they are abundant in Iraq. Also leave of *Ocimum basilicum* rich in active ingredients which have antiviral activity by different mechanisms (4). Recent experimental investigation by Lee-Huang and co-workers (5) found that OLE (olive leaf extract) shows strong anti- HIV activity. That OLE inhibits acute infection (new virus infection) and cell-to-cell transmission (virus replication) of HIV-1 (5 and 6). However, this effect is usually to varying degrees depending on the mechanism in which it operates, and depending on the type of plant extracted and extraction method and the amount of the administered dose. Due to the lack of studies available in Iraq on these plants and their content of compounds with antiviral activity, present work aimed to highlight on them.

Materials and Methods

Fresh *Ocimum basilicum* leaves were purchased from a local market in Baghdad during August to November and also *Olea europaea* leaves were picked from the local

olive trees during November to February. The plant classification was done in the Ministry of Agriculture/ State Board for Seeds Testing and Certification S.B.S.T.C in Abu Graib /Baghdad. Dried leaves were used for preparation ethanolic extract. The antiviral effect of extract of plant leaves, New castle Disease virus was local isolated strain from one of the infected fields with Newcastle disease virus in Al- Anbar province, was identified by heamagglutination inhibition test and serum neutralization test. The chicken embryo fibroblast monolayer was prepared as the method (7). Newcastle virus was titration on cell fibroblast in Microtiter plate to determinate Infected Dose of 50% Tissue Culture ($TCID_{50}$). The titration was calculated as (8).

Organic solvent extraction of the *Ocimum basilicum* and *Olea europaea* leaves was carried out by using ethanol (95% ethyl alcohol) which is considered as very effective in extracting the active ingredients of the plant according to (9). This was done by using magnetic stirrer, 50 mg of plant leaf powder with 500 ml of 95% ethanol was put inside the flask. The extraction was carried out for 24 hours by heating temperature that kept the solvent at 40-45°C for 24 hours at room temperature. The solution has been filtered through a Belgium filter paper, then the extract was dried by using an incubator under 38-40°C for dryness of the extract. The final extract was kept frozen at –20°C until use.

The chemical tests were carried out on the plant powder and its ethanol extract by using standard procedures to identify the constituents as (10). To conducting the antiviral properties, maximum nontoxic concentration of extract was determined in 24 hour grown monolayer CEF culture. The extract was diluted so as to contain 5000, 2500, 1000, 500, 250, 125, 100, and 50 µg /ml of extract in maintenance medium. 1 ml of each dilution was inoculated to CEF culture in a six wells culture plate and incubated at 37°C in the presence of 5% CO2.

The toxic effect of each concentration was observed under microscope at 12 hour intervals up to 48 hrs. Highest dilution showing any degenerative changes/ cytopathogenic effect (CPE) in cell culture was considered as cytotoxic concentration of the extract (4). Two different concentrations less than the nontoxic concentration i.e. 500 and 250 µg/ml used to determine the antiviral effect of extract of plant leaves. The monolayer cultures were challenged with ND virus having 2×10^{-7} $TCID_{50}$ with maintenance medium.

The different concentrations (500, 250 µg /ml) of the extract of each plant were added to NDV in test tube and later incubated at 37°C for one hour in the incubator. Into each well of a 24-well Microtiter tissue culture plate was added in duplicate 1 ml of virus-extract mixture inoculated in chicken fibroblast, adsorption at 37°C for 1 hour, maintenance media added and covered with adhesive paper the cells were incubated for 5 days in CO_2 incubator and monitored with the aid of an inverted microscope for CPE compared with control cells (fibroblast cells with extracts, fibroblast cells with a virus, fibroblast cells with DMSO and normal cells) (11).

The complete monolayer fibroblast were inoculated with 0.5 ml of plant extract at different concentration (500, 250 µg /ml) and incubated at 37°C for 1 hour. The extracts were removed after incubation to prevent any interaction with Newcastle virus, 0.2 ml of Newcastle virus was added to the falcons incubate for 1 hour for adsorption to the cells and then added maintenance media and incubate at 37°C in incubator for 5 days and monitor daily for CPE (11).

The complete monolayer were dispensed into falcon and inoculated with 0.2ml of virus and incubate at 37°C for adsorption for 1 hour, after one hour plant extract with different concentration (500, 250 µg /ml) with maintains media was added and incubated for 5 days and monitor daily for CPE (11). non-toxic concentrations (500, 250 µg /ml) used to determine the antiviral effect of leaf plant extract, conducted three treatment on of fibroblast cells in 96 a micro titer tissue culture plate.

Results and Discussion

The results of the local isolated virus had mortality rate 100% the embryos when inoculated into allantoic sac in a period of less than 48 hours, compared with the control

group. The lesions observed on dead embryos were congestion and severe hemorrhage (Fig. 1). Compared to the embryos control group that did not show any congestion or hemorrhage. The results showed that the isolation of virus in embryo of chicken eggs that reported death less than 48 hours indicate to virulence of the isolated virus, especially the virulent strain caused the dead of embryos eggs duration of less than 60 hours as pointed out by researcher Alexander (12). Active components in *Ocimum basilicum* and *Olea europaea* plant leaves 95% ethanolic crude extract and results of detection were listed in the (Table, 1).

Figure, 1. The lesions on dead embryos of chicken eggs that inoculated with virulent Newcastle virus (A) compared to control group (B) after 48 hours.

Table, 1: Phytochemical analysis of *Ocimum basilicum* and *Olea europaea* leaves extract.

Active Compounds	Type Reagent	Result of *O. basilicum* and *O. europaea*
Tannins	1. Lead acetate 1% 2. Ferric chloride 1%	(+) white gelatin ppt. (+) Blue-Green color
Glycosides	Benedict reagent	(+) red precipitate
Saponnins	1. Shaking of the extract 2. mercuric chloride	(+) thick foam (+) white precipitate
Coumarins	NaOH + filter paper + source of ultraviolet rays	(+)yellow-greenish shiny
Flavononid	Ethanol+ KOH	(+) Yellow Color
Alkaloid	Picric reagent	(+) yellow precipitate
Resins	95%ethanol + 4%HCL	(+) turbidity
phenols	Ferric chlorid 1%	(+) bluish-green color
pH		O.basilicum = 6.3 O.europaea = 4.22

The results of this study showed that the plant investigated has potential in the management of Newcastle disease. The ethanolic extract of leaves of *Ocimum basilicum* and *Olea europaea* showed promise in inhibiting NDV. This is essentially due to the pharmacologically active substances as revealed by the phytochemical analysis (Table 1). Alkaloids, flavonoids, saponins and tannins found in the plant are regarded as novel antiviral agents (13). Initially the nontoxic dose of extract was assessed and lowest dose showing the cytopathic changes in chicken embryo fibroblast cell culture was 1000 µg /ml in the maintenance medium (Table, 2 and Fig. 2). Thus all the concentrations of 500, 250, 125, 100 and 50 µg /ml which were less than 1000 µg /ml were considered nontoxic concentrations and were used for antiviral activity (4 and 14). The antiviral effects were assessed on the basis of occurrence of changes in the CEF monolayer culture and the $TCID_{50}$ titers of ND virus in the supernatant of culture. The viral reduction titer was found range between 10^{-6} to 10^{-1} with 500 µg /ml of *Olea europaea* and *Ocimum basilicum* extract as compared to 10^{7} with virus control (Table, 3 and 4). Simultaneously it also prevented the CPE of the ND virus. The virus infection produces cytopathic effects on chicken embryo fibroblast monolayer culture. Any reduction in the changes or absence of CPE is supposed to be protective effects of extract. At the same time the reduced $TCID_{50}$ titer is also indicative of inhibition of viral growth. Kumar *et al.* (15) and Sikader *et al.* (16) reported the antiviral effect of different extracts on infectious bursal disease and Newcastle disease viruses. In the *in vitro*-inoculate treatment extracts where the virus was pre-inoculate with the extract before administration, inhibition of virus growth was observed with two concentration (500 and 250 µg/ml). Previous studies earlier revealed the possibility of virus inactivation when a potent

antiviral select is incubated with the virus at controlled conditions (17).

The possibility that the extract interferes with the neuraminidase-haemagglutinin sites necessary for attachment and penetration of the virion into the living cell is very likely. In the pre-inoculate treatment extracts when the cell line was pre-treated with the plant extract prior to infection with the virus. Here, the degree of virus inhibition was also good. Scientists have earlier explained that if the receptor sites of susceptible hosts are bound or altered prior to virus infection, the ability of the virus to attach and penetrate the living cell would be greatly reduced (18).

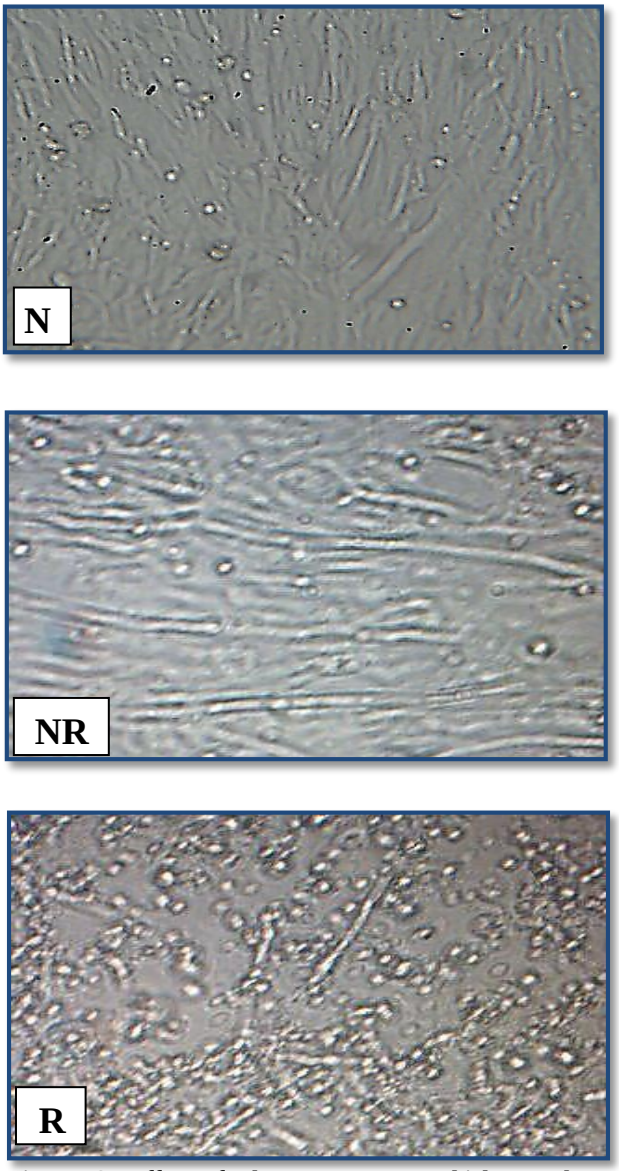
In the post-inoculate treatment extracts. Here, the virus was made to infect the cell before introduction of the plant extract. Although at 500 and 250 µg/ml concentration of plant extracts, it inhibited virus growth, it generally did not show the degree of potency and sensitivity with smaller concentrations; as it was the case with the other two (Table, 5). This presupposes that the extracts may exhibit minimal antiviral activity once the virus has attached, penetrated and uncoated in the cell. It could be conclude that the ability of the extracts to alter virus replication in stages subsequent to attachment and penetration of host cell is not as effective as prior to the aforementioned stages. Although a greater degree of success may be achieved with higher extract concentrations.

Antiviral agents are known to interfere with virus replication at different stages of virus replication (18). Although three-inoculate processing extracts of both extracts have potentials for antiviral activity against NDV, the *in vitro* and pre- inoculate treatment extract seem to have more of the antiviral components than the post-inoculate treatment extract. This is based on the role of both extracts as immunomodulator has been thoroughly studied and well established (19 and 20). According to the above results, the present study demonstrated that the inhibitory effect of *Olea europaea* and *Ocimum basilicum* leave extract is higher respectively in pre viral infection and during infection.

Table, 2: The effect of leaf plant extracts on fibroblast cells.

Extracts (µl /ml)	Morphology of fibroblast cell
5000	R
2500	R
1000	NR
500	N
250	N
125	N
100	N
50	N

R: cells were rounded (toxic) N: cells were normal (spindle shape) (nontoxic), NR: cells were normal with rounded cell (Low toxicity)



Figure, 2: Effect of plant extracts on chicken embryo fibroblast culture. (N) cells were normal (spindle shape) (nontoxic) (NR) cells were normal with rounded cell (Low toxicity) (R) cells were rounded (toxic).

Table, 3: The effect *in vitro*-inoculate treatment extracts on titer NDV.

Plant species	Extracts (µg /ml)	Control Virus	*In vitro*-inoculate treatment	Reduction titer
O.basilicum	500	10^{-7}	10^{-2}	10^{-5}
	250	10^{-7}	10^{-3}	10^{-4}
O.europaea	500	10^{-7}	10^{-1}	10^{-6}
	250	10^{-7}	10^{-2}	10^{-5}

Table, 4: The effect Pre-inoculate treatment extracts on titer NDV.

Plant species	Extracts (µg /ml)	Control Virus	Pre-inoculate treatment	Reduction titer
O.basilicum	500	10^{-7}	10^{-2}	10^{-5}
	250	10^{-7}	10^{-3}	10^{-4}
O.europaea	500	10^{-7}	10^{-1}	10^{-6}
	250	10^{-7}	10^{-2}	10^{-5}

Table, 5: The effect Post-inoculate processing extracts on titer NDV.

Plant species	Extracts (µg /ml)	Control Virus	Post-inoculate treatment	Reduction titer
O.basilicum	500	10^{-7}	10^{-4}	10^{-3}
	250	10^{-7}	10^{-4}	10^{-3}
O.europaea	500	10^{-7}	10^{-2}	10^{-5}
	250	10^{-7}	10^{-3}	10^{-4}

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الفعالية المضادة للفايروس للمستخلص الكحولي لأوراق نبات الريحان والزيتون في خارج الجسم على فايروس نيوكاسل الدواجن

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الخلاصة

أجريت هذه الدراسة لمعرفة تأثير المستخلص الكحولي لأوراق الزيتون والريحان لدراسة النشاط المضاد للفايروسات ضد فايروس النيوكاسل وتأثيرها على خلايا الارومة الليفية واظهَر ان تراكيز (50 و 100 و 125 و 250 و 500) ماكرو غرام/مل للمستخلص الكحولي لأوراق الزيتون والريحان في الوسط الحافظ غير سامة. حيث معرفة التأثيرات المرضية لفايروس النيوكاسل على خلايا الارومة الليفية احادية الطبقة لذلك استعملت هذه تركيز للكشف عن نشاط مضاد للفايروسات لأوراق الزيتون والريحان فضلاً عن معاملته خارج وقبل وبعد الحقن المستخلص النباتية في الخلايا الارومة الليفية لتكوين فكرة عن انخفاض المعيار الفايروس في خلايا الزرع النسجي، عدم وجود آثار الاعتلال الخلوي في خلايا النسجي أحادي الطبقة وانخفاض معيار الفايروس يستعمل كمؤشر على نشاط مضاد للفايروسات لمستخلص اوراق الزيتون والريحان. أظهرت معاملة مستخلص النباتي لأوراق الزيتون والريحان خارج وقبل وبعد الحقن في خلايا الارومة الليفية امتلاكها نشاط مضاد لفايروس النيوكاسل بتركيز (500,25) ماكرو غرام/ مل. وجد أن خفض عيار الفايروس تتراوح ما بين 10^{-6} الى 10^{-1} بتركيز 500 ماكرو غرام| مل لمستخلص الزيتون والريحان مقارنتا 10^{-7} لفايروس السيطرة.

الكلمات المفتاحية: اوراق الزيتون، اوراق الريحان، المستخلصات النباتية، فايروس النيوكاسل، خلايا الارومة الليفية.