INTERNATIONAL JOURNAL OF SCIENCE AND NATURE

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ANTIVIRAL EFFECT OF *MYRISTICA FRAGRANS* ON CALF ROTAVIRUS (*IN VITRO*)

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ABSTRACT
This is the first study in Iraq which was carried out to isolate rotavirus in chicken embryonic fibroblast cell culture (CEFCC) and inoculation the virus in chicken embryonic egg for manifested the pathological changes in chicken embryonic egg. Also to investigate the antiviral activity of *Myristica fragrans (Mf*) extract on rotavirus in CEFCC. The results of infected cell culture showed the cytopathic effect (CPE) which include rounded cell, giant cell formation and completely detachment the cells from the surface of falcon also the pathological changes in chicken embryonic egg that include haemorrhages of subcutaneous tissues of chicks and congested of chorioallantoic membrane. Rotavirus A was detected by real time PCR in calf diarrheal samples, isolated virus in CEFCC and inoculated virus in chicken embryonic egg. The result of co-incubation and pre-incubation of CEFCC with M*f* extract was inhibited the GCPE of calf rotavirus at concentrated of G0.5 μg/ml, but the post-incubation o*f* CEFCC with Mf extract was inhibited this virus at concentrated h2.5μg/ml.

KEYWORDS: *Myristica fragrans* calf rotavirus in *vitro.*

INTRODUCTION

Myristica fragrans(*Mf*) houtt belongs to the *Myristicac eae* family and has a broad spectrum of pharmo-logical effects. The most important part of the plant in pharmacological activity and in commerce is dried kernel (seed), the nutmeg, and is very effective against various animal and plant bacteria, fungi and harmful viruses, insects and snails[1]. The study of Grover *et al*. [2] showed the nutmeg is a good antidiarrheal effect and revealed that the extracts of nutmeg show a good antidiarrheal effect, with a significant selective property *Rotavirus* (*RV*) is the main causes of diarrhea in human[3], calf and other species of animals[4]. It is a genus belong to *Reoviridae* family[5]. *RV* is double-stranded RNA and the nucleic acid is surrounded by a three-layered icosahedral protein capsid, non enveloped[6,7]. Viral particles are upto 76.5 nm in diameter. Although rotavirus was discovered in 1973 by Ruth Bishop and her colleagues by electron micrograph images [8]. Rotavirus is transmitted by the fecal-oral route, via contact with contaminated hands, surfaces and objects [9]. It infects and damages the cells that line the small intestine and causes gastroenteritis. Rotaviruses cause economic loss to farmers because of costs of treatment associated with high morbidity and mortality rates[10]. There is evidence that animal rotaviruses can infect humans, either by direct transmission of the virus or by contributing one or several RNA segments to reassortants with human strains[11,12]. Various molecular techniques have been exploited for the development of highly sensitive and rapid assays for the detection of causative agents of viral gastroenteritis (13). PCR have been shown to have a higher sensitivity than other test for detection rotavirus and identify all species and serotypes of rotavirus [14].

The Dhamaik, *et al* [22] isolated the rotavirus in Mad in darby bovine kidney cells. Also the study of Gonclave *et al*.[39] reported that the activity of antirotavirus of *Mf* extract inhibited the human rotavirus in *vitro* at 90%. Al-Gburi *et al.*[40] confirmed the antiviral activity of *Mf* extract when was used to infect the calf with rotaviral diarrhea and lead to inhibit this virus at 90-100%. The aim of this study was to manifest the CPE of rotavirus in CEFCC and pathological changes in chicken embryonic eggs also was to investigate he activity of antirotaviral from *Mf* extract against calf rotavirus in CEFCC.

MATERIALS & METHODS

Collection the fecal samples

Fecal samples were suspended in 10% PBS, clarified by centrifugation at 2000 rpm for 15 min. And supernatants were collected and stored at -20 till further use. All clinical samples were detected by using commercially available latex agglutination LA test. Titration of rotavirus by Haemagglutination (HA) test: micro method was performed by using 0.5% chicken RBCs [15]. Cultivated of rotavirus in chicken embryonic egg by using allantoic method at 9 days. Isolation of rotavirus in chicken embryonic fibroblast cell culture (CEFCC): The CEFCC was prepared in virology laboratory/College of Veterinary Medicine /University of Baghdad [16]. Isolation of calf rotavirus was performed as per method of Saravanan *et al.* (2006)[17]. Briefly, the rotavirus +ve supernatant fluids were filtered through 0.45 μm and filtrates were mixed with an equal volume of minimum essential medium containing 2% fetal calf serum and 10 μg/ ml trypsin and incubated at 37 for 60 min, finally inoculated in CEFCC

Identification of isolated rotavirus A by Real Time PCR-One Step:

This amplification kit has been manufactured by Genekam Biotechnology AG, Germany to detect rotavirus A and is based on fluorogenic dyes.

Extraction of viral RNA using QIAamp viral RNA kit: RNA extracted from fecal samples, two isolated virus in CEFCC and allantoic fluid in chicken embryonic egg by fallowing program:

- Add 140$\mu\ell$ of sample, add 560 $\mu\ell$ of buffer AVL and add 560$\mu\ell$ of absolute ethanol (between each step vortex for 10 min.
- Transfer 630 $\mu\ell$ of the above mix to spin column and centrifuge for 1 min. at 8000 rpm.
- Discard filtrate tube, place column in a new collection tube and add 500 $\mu\ell$ AW1 buffer and centrifuge for 1 min. at 8000 rpm.
- Discard filtrate tube, place column in a new collection tube and add 500 $\mu\ell$ AW2 buffer and centrifuge for 1 min. at 14000 rpm.
- Discard filtrate and reuse the collection tube for another spin at max speed for 1 min.
- Discard filtrate tube, add 40 $\mu\ell$ AVE buffer and incubate for 1 min. and centrifuge for 1 min. at 8000 rpm.
- Finally store the extracted RNA at -70 .

Real Time PCR – One Step: for RT-PCR, preparation of 7$\mu\ell$ of solution A + 10$\mu\ell$ of solution B+ 1$\mu\ell$ of solution Y for each samples, then added 2$\mu\ell$ of extracted viral genome, 2$\mu\ell$ of D1 to +ve control and 2$\mu\ell$ of D2 to –ve control, after that mix all tubes and centrifuged at 8000 rpm for 20sec. finally microtubes (96-well reaction plate) must be in contact with metal block of thermocycler. The amplification was carried out using the fallowing program: 3600sec. at 42 ,600 sec. at 70 and 15 sec. at 95 , 60 sec. at 58 .

1. Extraction of *Mf* plant has been extracted by using methanol (70%), (1:5) which is considered as very effective in extracting the active ingredients of the plant according to method described by Sonavane *et al.* (18).
2. Phytochemical screening of the *Mf* extract was carried out according to the methods described by Harborne [19].
3. Preparation of stock solution of *Mf* extract was done by dissolving 1g of *Mf* extract in 5 ml of maintenance media. A concentration of 0.5, 1, 2, 2.5, 5, 10, 25, 50, 100, 125, 150 and 200 μg / ml was prepared for in the *vitro* injection.
4. Cytotoxicity assay: The different concentrations of extract (0.5, 1, 2, 2.5, 5, 10, 25, 50, 100, 125, 150 and 200 μg / ml) were added to CEFCC in 24 well plate tissue culture and incubated at 37 for 3 days and monitored the morphological changes compared with control untreated cell everyday (20).
5. Inoculation treatment of CEFCC with different concentrations of *Mf* extract (co-incubation, pre-incubation and post-incubation)

Co-incubation :1ml of each concentration of *Mf* extract mix with 1ml of virus and incubated at 37 for 1 h., then inoculated on CEFCC, adsorption occur at 37 for 1 h. and added maintenance media, finally incubated at 37 for 3 days and noticed daily the morphological changes

Pre-incubation : 1ml of each concentration inoculated on CEFCC, incubated at 37 for 1 h., *Mf* extract removed, inoculated 1ml of virus, adsorption occur at 37 for 1 h. then added maintenance media, finally incubated at 37 for 3 days and noticed daily the morphological changes.

Post-incubation : Inoculated 1ml of virus on CEFCC, adsorption occur at 37 for 1h. then added maintenance media containing each concentration of *Mf* extract, finally incubated at 37 for 3 days and noticed daily the morphological changes.

These treatments compared with normal cell (uninfected cell), infected cell with virus only and treated cell with plant only [21].

RESULTS & DISCUSSION

The supernatant of collected fecal samples from diarrheic calves screened by LA test were +ve for rotavirus and the results of HA test showed ability to detect the virus to agglutinate chicken RBCs, it indicate positive results, the titer of detected virus was 2048. The pathological changes of the virus which include no death of chicks, haemorrhage of chicks and congested, thickness of chrio-allantoic membrane (fig.1).

1- Isolation of rotavirus on chicken embryonic fibroblast cell culture (CEFCC): the infected cell showed

a- CPEs started after 24 h. (P.I.) (fig.2) characterized by focal cell rounded,clumped and formation of vaculated cell, but no cellular changes were noticed in control CEFCC

b- CPEs after 48 h. (P.I) became more pronounced of large syncytia, floating and detachment of the cells from surfaces of falcons.

c- CPEs after 72 h. (P.I) showed moth eaten appearance.

d- CPEs after 96 h. (P.I) revealed completely detachment of the cells from the surface of falcons .

In Iraq this study is the first one to isolate calf rotavirus by using chicken embryonic fibroblast cell culture (CEFCC). The results of cytopathic effects (CPEs) in CEFCC agreed with the results of Dhama et al., (22) However, the mentioned study used the madin darby bovine kidney (MDBK) cells line.

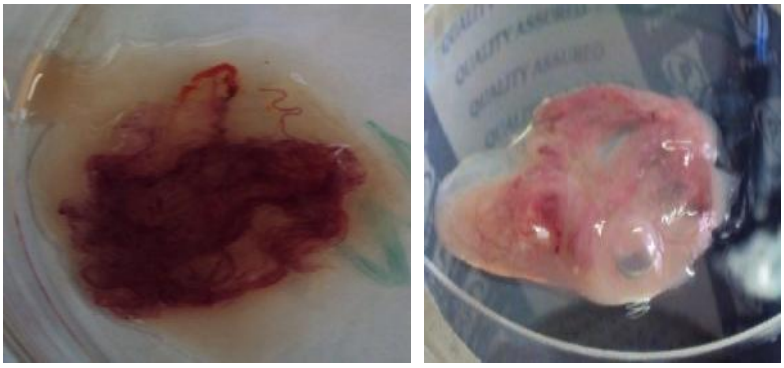

FIGURE 1: Congested of chorioallantoic membrane **A:** infected **B:** non-infected

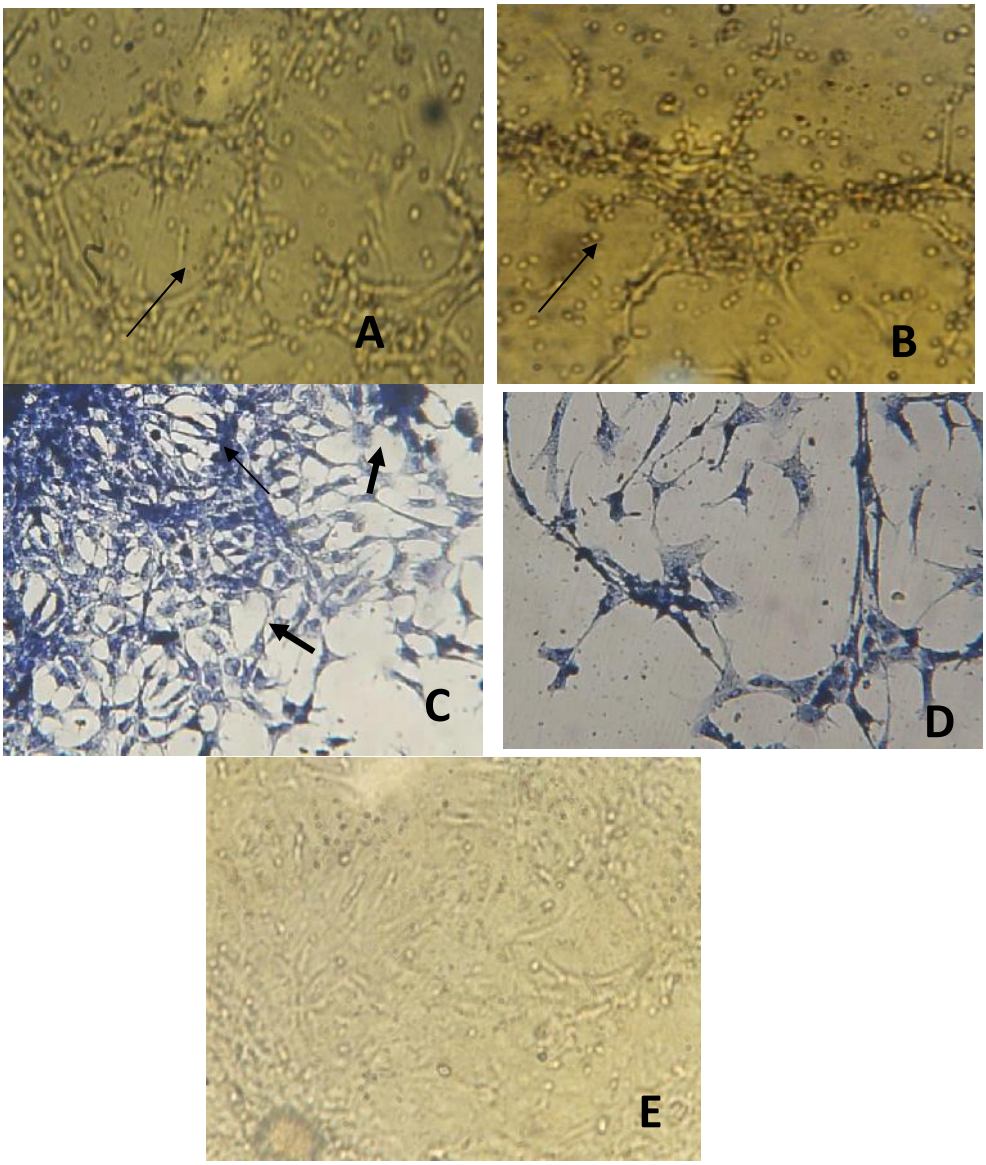

Figure (2): A: Infected CEFCC with calf rotavirus after 24 h. P.I., focal cell clumped, rounded and vacuolated cell formation, 100x. **B:** Infected CEFCC with calf rotavirus after 48 h. P.I., syncytia formation, floating and detachment of cells from surfaces of falcons, 100x. **C:** Infected CEFCC with calf rotavirus after 72 h. P.I., moth eaten appearance, 100x. **D:** Infected CEFCC with calf rotavirus after 96 h. P.I., completely detachment of the cells from the surface of falcons, 100x. **E:** Normal CEFCC 100x.

The results of Real Time-PCR: As a highly specific and sensitive method, RT-PCR technique was used to confirm the current viral isolates. All 4 samples (fecal samples, two isolated virus and inoculated virus in chicken embryonic egg) were found positive by this technique, and those results were compatible with our previous finding of the cell culture inoculation, latex agglutination and Elisa test.

The RNA was detected in four samples of calves rotavirus and ranges reading of the threshold cycler time value (CT v) by using real time PCR specific to calf rotavirus. It was recognized as positive samples in the range of standard curve, with CT v (< 32) the results as seen in (fig.3) that showed the positive samples among reading of (CT v) with positive control in calves.

Several studies conducted previously conclude that Real time PCR as a very sensitive test for the detection of rotavirus A. Saravanan et al. (23) revealed the rotavirus A that is viral pathogen and most common cause of gastroenteritis by nested-multiplex PCR in neonatal calves in India, also Basera et al. (24) was detected the rotavirus A from cattle and buffalo calves with diarrhea which were examined using RT- PCR and RNA-PAGE. Anamul et al., (25) detect the rotavirus A in diarrheic bovine calves by RT-PCR assay.

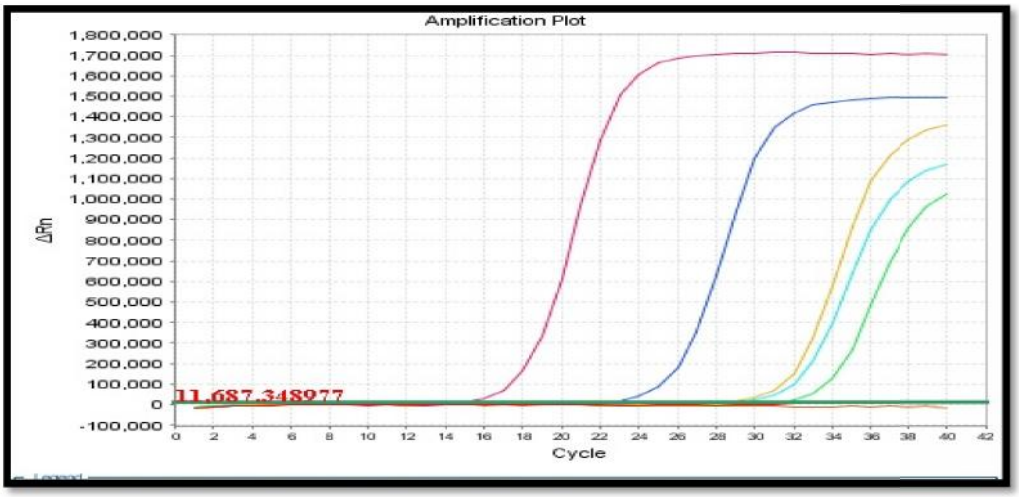

FIGURE 3: The four positive fecal samples (isolated virus and inoculated virus in chicken embryonic egg)

The results of methanolic extract of nutmeg gave dark brown oily sticky at 10% according to Banso[26]. Several studies showed 80% methanolic extract at 3.34% [27] and 50% ethanolic extract (21.20%)[28]. These differences of results may be due to the difference in the origin and quality of the seeds or different parts of the plant, or due to the use of different proportions of water –alcohol mixture, and time for extraction.

The results of phytochemical screened in methanolic extract of nutmeg revealed the presence of eight components. Saponins, alkaloids, flavonoids and glycosides were agreed with Olaleye[29], also the presence of tannins and phenols w agreed with the results obtained by some studies [28, 30], as well as the presence of the terpenes and resins.

The effect of antiviral effect on rotavirus in CEFCC was listed in lower table, also the isolated virus was +ve when tested by LA test and (512–2048) tittered by HA test.

TABLE 1: anti-rotaviral effect of *M.F.* extract on CEFCC

Concentration of *M.F.* extract μg/ml	Cytopathic effects of rotavirus		
	Co-incubation	Pre-incubation	Post-incubation
0.5	-ve	-ve	+ve
1	-ve	-ve	+ve
2	-ve	-ve	+ve
2.5	-ve	-ve	-ve
5	-ve	-ve	-ve
10	-ve	-ve	-ve
25	-ve	-ve	-ve
50	-ve	-ve	-ve
100	-ve	-ve	-ve
125	-ve	-ve	-ve
150	-ve	-ve	-ve
200	-ve	-ve	-ve
-ve = Like the control CEFCC (uninfected cell)			
+ve = CPE (infected cell with virus)			

These results of antirotaviral of *Mf* extract in CEFCC against *RV* may be due to the presence of some phytochemicals components that interference with either viral replication or capacity to bind to permissive cells.

The results of co-incubation may be belonged to phenolic compounds which may acts directly by interaction with virus particles at early stage of infection and block the liberation of its nucleic acid that lead finally to stop the virus multiplication (31) and shown to prevent viral replication, inhibition of virus attachment to and penetration into cells, and virucidal effects [32]. The results of pre-incubation may be due to flavonoids compounds which could inhibited the penetration of *RV* in CEFCC and affect on the enzymes responsible for their replication[33,34] which include the ability to inhibit viral polymerase, binding of viral nucleic acid or viral capsid proteins [34,35], Also this could be attributed to anther mechanism that lie in the inactivation of *RV*, as shown with enhancement of cell survival after pre-incubation of rotavirus with *Mf* extracts. The results of post-incubation may be due to tannins compounds have inhibited both of viral cytopathic effect and expression of antigen[36] and the effect of toxins and it's binding with protein led to enzyme inactivity[37], also may be due to alkaloids compounds have ability to inhibit protein synthesis during viral replication [38].

These results agreed with the results obtained by Goncalves[39] who reported that *in vitro* anti rotavirus activity from the *Myristica fragrans* extract was inhibited the human rotavirus at a percentage of 90%. Also the results agreed with Al-Gburi [40] who detected the inhibition of calf rotaviral diarrhea *in vivo* (calf) at 90-100%. From this sought was concluded, the *Mf* extract have highly significant inhibitory effects against cyto pathic effects of *RV* and the best anti-rotaviral activity at lowest concentrations (0.5 μg/mL).

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