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POLYPHENOLIC WEALTHY MORINGA LEAVES EXTRACTS AS ANTI-COXSACKIE B VIRUSES (COX-BV)

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Naglaa A.Saif[1], Othman[2] B.A., Mohamed[3] A.F. and El-Dougdoug[2] Kh.A.

1. General Authority for Health Insurance Hospital, Sednawy, Cairo, Egypt.
2. Agric. Microbiology Dept., Fac. of Agric., Ain Shams Univ., P.O. Box 68, Hadayek Shobra 11241, Cairo, Egypt
3. Holding Company for Biological product and vaccines, Giza, Egypt.

*Corresponding author : naglaa_Abozied@yahoo.com

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ABSTRACT

The antiviral role of *Moringa*oleifera leaves extracts ((chloroform (CL), Ethyl acetate (E.A), methanol 80% (M) and n.butanol (n.b) to control Coxsackie B (COX-BV) viral infection was monitored in vitro compared with human interferon alpha (IFNᾰ-2a) in order to evaluate the antiviral activity of moringa leaves extracts. The phenolic compound was given higher conc. in E.A. extracts. Cytotoxicity was determined for *Moringa* leaves extracts on viability of HEP2 cell using MTT assay. The 98µg/ml concentrations for four extracts were considerably non-toxic for HEP2 cell line culture. Antiviral activity in *Moringa* extracts may be attributed to phenolic compounds and / or as a result of stimulation of COX-BV-sensitive HEP2 cells to express MX protein. Antiviral activity of *Moringa* leaves extracts compared with IFNᾰ-2a to cell lines against COX-BV viral infection was determined by assessment of the COX-BV virus infectivity titer declining rate and relative residual living cell count using End Point Assay, using three ways pre-treatment, co-treatment and post-treatment cell line treated with *Moringa*extracts. It was found that the extract acetyl acetate was better as an MX gene expression level compared with its level in control cells. It was concluded that *Moringa*leaves extracts have antiviral activity against COX-BV.

Key words: Moringa Oleifera extracts, phenol compound, IC50, Cytotoxicity, Antiviral activity,MX gene.

INTRODUCTION

Since many thousands of years man-kind used the plants as source to eliminate or cure illnesses because the plants' constitute a source of novel chemical compounds which are used in medicine as an antimicrobial, Antioxidant, anticancer, antiseptic, anti-inflammatory, antiparasitic and other applications.**(Atanasov et al 2015, Greenwell and Rahman, 2015 and Yuan et al 2016).**

Moringaoleifera Lam is recognized as a vibrant and affordable source of phytochemicals, having potential applications in medicines, functional food preparations, water purification, and biodiesel production. The multiple biological activities including Antimicrobial, antiproliferation, hepatoprotective, anti-inflammatory, antiperoxidative, the folk medicinal uses of *M. oleifera* are attributed to the presence of functional bioactive compounds such as phenolic acids, flavonoids, alkaloids, phytosterols, natural sugars, vitamins, minerals, and organic acids. These phytochemicals for antiviral activities have assumed greater importance in the last few decades. Which, may be explained on basis of their antioxidant activities, scavenging capacities, inhibiting DNA, RNA synthesis, inhibition of the viral entry, or inhibiting the viral reproduction **(Naithani et al 2010, Saini et al 2016, Brilhante et al 2017, Kapoor et al 2017, Abd El-Hack et al 2018 and Chodur et al 2018)**. The low molecular weight cationic proteins of *M. oleifera* were extracted in water from its seeds potent antimicrobial and coagulant properties **(Lin et al 2018).**

In recent years it has been recognized that viruses are an important cause of food-borne diseases. Enteric viruses are the principal agents for outbreaks of food related illness outbreaks worldwide and are responsible for estimation of about 40-50 million illnesses each year in the United States (**Todd and Greig, 2015 and Neethirajan et al 2017**). About 40 million (80%) of the illnesses attributed to Noro viruses, Hepatitis A and E virus-

es, Adenovirus (types 41 and 42), Rotavirus, Enterovirus that include Coxsackie B viruses, as well as other RNA viruses groups. (**Bosch et al 2018)**.

Nature has endowed us with number of medicinal plants which possess magical therapeutic agents that can be utilized for number of diseased states. *Moringa*oliefera is a plant belonging to a family Moringaceae which is hugely cultivated throughout the world. Common names of this plant are drumstick tree, horse radish tree and suhanjna. All parts of the plant are immensely nutrimental including bark, leaf, root, seeds, gum, pod, fruit and flowers **(Lin et al 2018, Oyeyinka and Oyeyinka, 2018, Rani et al 2018, Saucedo-Pompa et al 2018 and Wang et al 2018).** Antiviral activity of *Moringa*oliefera has been stated earlier against much publicized diseases i.e. HSV, HIV, and EBV, influenza, yellow fever virus and polio virus **(Younus et al 2015).**

Coxsackie B viruses, belongs to the family picornaviridae, are non-enveloped viruses which have the ability to survive harsh environments. Infection proceeds via the fecal/oralroute, and hence virion stability in the acidic environment of the stomach becomes a necessity for efficient transmission. Type B coxsackie viruses include six serotypes, each being associated with acute disease in humans, including acute viral myocarditis and pancreatitis. While Coxsackie B viruses is generally regarded as a lytic virus, and emerging evidence suggests that persistent infection can be established which may be responsible for chronic inflammation with in target organs. **(Sin et al 2015 and Afrose, 2017).** The serotypes of Coxsackie B viruses are able to replicate in pancreatic β cells. Infection can lead to beta-cell apoptosis which increases the risk of insulitis. Sothat, many publications have suggested a link between early Coxsackie B viruses infection and insulin-dependent diabetes (IDDM) **(Christen et al 2012, Laitinen et al 2014, Sin et al 2015 and De Beeck and Eizirik, 2016).** Coxsackie B virusesinfections have worldwide distribution. The infection occurs in all age groups but young children, infant and immune compromised groups are at high risk for the complications **(Afrose, 2017).**Coxsackie B viruses are usually spread to infants through perinatal transmission. However, more severe cases of Coxsackie B viruses are spread through transplacental transmission. Common symptoms of neonatal Coxsackie B viruses infection in children include meningitis and/or encephalitis. Coxsackie B virusesis able to infect the brain and spinal cord and cause inflammation**. (Marc et al 2011).**

In view of the high, antiviral value of this plant**.** This research aims to use its leaves extracts as antiviral agents against Coxsackie B viruses in vitro.

MATERIALS AND METHODS

The work of this study was carried out in Virology lab, Dep. of Agri.Microbiology., Fac. of Agric.Ain Shams Univ., Sednawy Hospital and R&D Sector, the Holding Company for Biological Product and Vaccines (VACSERA).

Plant sample

Moringaoleifera Lam fresh leaves were collected from virology botanical garden of Fac. of Agric. Ain Shams Univ. during October, November and December 2016. The leaves samples were washed properly and dried at 40°C for 4hr.

Virus sample

A Coxsackie B virus (COX-BV) it was isolated and characterized by **Seif et al (2016).** COX-BV was propagated in HEp-2 cell line (ATCC). Virus infectivity titer was determined according to **Reedand Muench**formula **(1938**)**.**

Cell line

Human epidermoid carcinoma larynx cells (HEp-2 cells) CCL-23 from American Type Culture Collection (ATCC) was kindly supplied from cell culture dept., the Egyptian Holding Company for Biological Product and Vaccines (VACSERA) as a confluent sheet according to the manufacturer protocol. HEp-2 cells were pre-cultured in 96-well tissue culture plates using E-MEM containing10% FCS and 1% Penicillin units /ml 1% streptomycin µg/ml for 24 h at 37°C in humidified incubation with 5% CO_2 (Thermo scientific® CO_2 incubator, Germany).

Recombinant interferon

Human recombinant IFN-2a (Laroferon) was purchased from F. Hoffmann-La Roche S.A. (Basel, Switzerland). Initial IFN-2a preparation, at $3x10^6$ U/mL.

Preparation of*Moringa* leaves Extracts

The dried leaves were homogenized into fine powder and extracted with differential solvents;

Chloroform, Ethyl either, methanol 80% and *n*.butanol according to **Mishra and Padhy (2013)** The extracts were filtered using Whatmann filter paper No. 1 and concentrated using rotary evaporator (Buchi, Flavil, Switzerland) at 45°C, dried and kept at 4°C till use. Extracts were redis solved in dimethylsulphoxide (DMSO), Sigma Aldrich - USA.

Estimation of total phenolic compound in *Moringa* leaves Extracts.

The total phenolic content of Chloroform, Ethyl acetate, *n*.butanol and methanol 80% extracts of *M. oleifera* was estimated in terms of gallic acid equivalence by Folin-Ciocalteau reagent by the method of **Singleton and Rossi (1965)** with slight modification according to **Luqman et al (2009)**.

Cytotoxicityassay of *Moringa*leaves extracts

Cytotoxicity of each extract was evaluated using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] MTT method according to **Takeuchi et al (1991) and Sieuwerts**, **et al (1995)**. Pre-cultured T.C plates growth medium was decanted and test extracts were dispensed to 24 hrs pre culturedcells. Treatment medium was decanted and 50 µL of MTT as 0.05 mg/ mlwere added to each well. The O.D were measured on a multiwell spectrophotometer (ELX-800,BioTek®, USA) at 570 nm and the average OD of wells for each extract dilutionswere calculated. Viability% was determined according to the following equation: Viab.% = average OD of test sample x 100 / average OD of untreated cell. Viability % was plotted against test extract concentrations and the highest non- toxic dilution was determined according to **Marini et al (1998).**

Evaluation of antiviral Activity

Application of the antiviral activity of the non-toxic tested Moringa extracts(Chloroform, Ethyl acetate, methanol 80% and *n*.butanol) as anti-Coxsackie B viruses were determined, in three ways, pre-treatment,post-treatment and co-treatment, using End Point Assay, according to **Vijayan et al (2004).** Parallel withIFNᾰ-2a (Basel, Switzerland). Confluent cell lines monolayer ($2x10^5$ cell/ml) were cultured in 96-well cell culture plates. On confluences, growth media were discarded. Coxsackie B viruses were 10 fold serially diluted in E-MEM media and tested extracts were 2 fold serially diluted in E-MEM media. The scored CPE was recorded and the virus titer was calculated using **Reed and Meunch (1938)** equation, the antiviral activity was evaluated in comparison to that achieved under the effect of IFN-2a.

Pre-treatment

10 TCID50/0.1 mlof the Coxsackie B viruses was titrated on cell lines post 24 hrs treatment with and without treated extracts. Plates were incubated at 37°Cthen microscopically examined using inverted microscope (Olympus®, Japan).

Post-treatment

The same tested were dispensed to infected cells post decanting of infectious medium. Plates were incubated at 37°C. The titre of virus was calculated by the same equation.

Co-treatment

The tested extracts were mixed with virus sample for2 hours at 37°C and dispensed to pre-cultured plates. The titre of virus was calculated by the same equation.

The antiviral activity was measured as the difference between test materials treated cell virus titer and non-treated cell virus titer. The depletion rate was expressed as the percentage loss in the virus infectivity titer.

Molecular biology

Hep-2 cells treated with non-toxic concentration of E.Aextract (200µg/ml)and IFN–α 2a (5µg /ml) and negative control cells were processed for RNA extraction to assess up regulation of MXprotein according to **themanufacturer instructions** (Qiagen RNA extraction/BioRadsyber green PCR MMX). The primers designed wer' [F] 5'-TTGAGGTGATGGTGAAAGACC-3', and [R]5'-TTGAGGTGATGGTGAAAGACC-3'.

RESULT

Total phenolic content

Plant phenolic constitutes one of the major groups of compounds acting as antiviral activity; it was worth determining their total amount (TPC) in the leaves extract of *M. oleifera.* Relative to gallic acid concentration the PAC recorded 15.38, 26.60, 14.22, and 21.68 mg/mL for CL, E.A, M and *n*. bextraction regimen respectively **(Fig.1).**

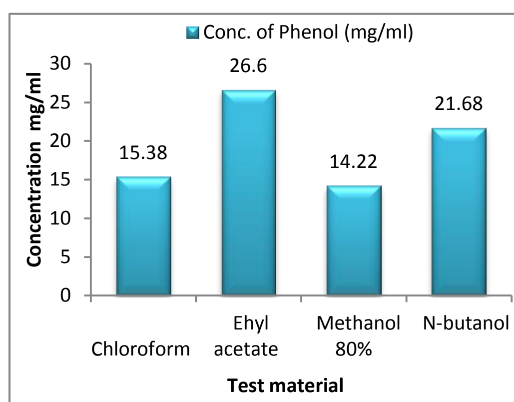

Fig. 1. Concentration of Phenolin extracts of *Moringaoleifera* leaves

Cytotoxic effect of test extracts using MTT assay

The toxicity of test extracts was assessed quantitatively using MTT assay. It was noticed that cytotoxicity induced was dose and cell type dependent. Regarding the untreated control it showed normal cell behaviors and morphology. Cell morphology showed alterations post treatment, producing detectable changes including various morphological abnormalities and alterations, including cell rounding and some areas devoid of cell sheet **(Fig. 2).**

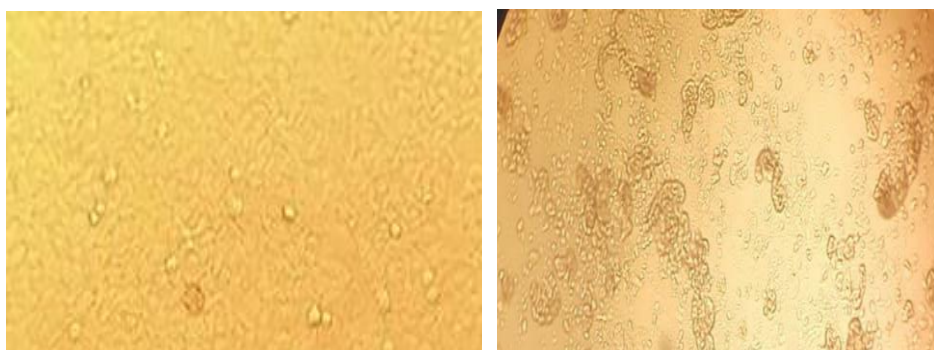

Fig. 2. Morphological change of Hep-2 cells,A:- Normal HEP2 cell, B:- Toxic HEP2 cell, showed, morphological abnormalities in toxic HEP2 cell compared with normal HEP2 cell at 100 magnification.

Cytotoxic assay of *Moringaoleifera* CL, ME, EA, and n.b leaves extracts showed that cell survival rates was relative to concentration and type of extract and viability increased as the concentration decrease. Thehigh decreased in the IC50 values of Cl, M, and EA more than in case of n.bextract (432, 565, 539 and 1079) respectively **(Fig. 3).** Concurrently there wasno difference of IC50 values among Cl, EA and M extract The relative viability % of living cell lines was determined using thiasolum blue stain (MTT- based colorimetric assay) recording 91.33 ,93.19%, 92.29% and 93.17 for CL, E.A, M and n.b extracts respectively, on HEP-2 cell lines at conc.100ug/ml **(Fig. 4).**

Antiviral of *Moringa* Leaves extracts

Regarding the antiviral potentials of test extracts, data showed that in the Pretreatment Cl extract showed the highest decrease of viral infectivity titer (68.3%) followed by EA extract (66.4%) . Both Cl and EA showed a higher antiviral activity than ME (21%) and N.(26.1%).

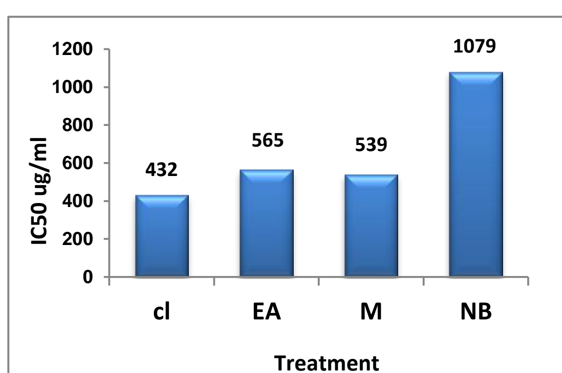

Fig. 3. IC50 values of test materials against Hep2 cell

cl - Chloroform extracts of Moringa Leaves
EA- Ethyl acetate extracts of Moringa Leaves
M- Methanol 80 % extracts of Moringa Leaves
NB- N-butanol extracts of Moringa Leaves

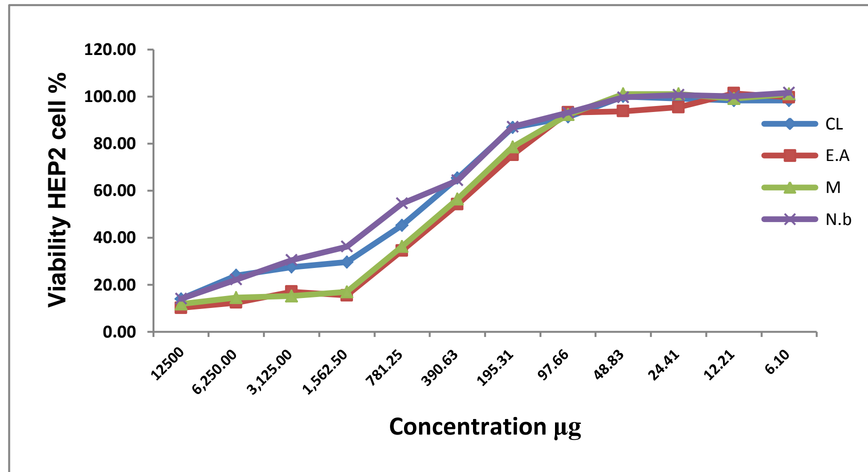

Fig. 4. Evaluation of viability % of plant extracts using MTT assay
Cl-:Chloroform extracts of Moringa Leaves
EA: Ethyl acetate extracts of Moringa Leaves
M: Methanol 80 % extracts of Moringa Leaves
n.b:-butanol extracts of Moringa Leaves

IN the mean time IFNă2a showed a higher antiviral potential than NB and ME. Also there was a difference of antiviral activity between Cl, EA and IFN. In the mean times evaluation of antiviral activity using the combination treatment method, as it was noticed that EA, M E and NB showed higher viral infectivity titer depletion that than in case of Cl extract and IFNă2a treatment. Finally it was noticed that IFNă2a showed a higher viral infectivity titer depletion than test extracts in case of post treatment arranged in the order of NB<CL< ME <EA respectively **(Fig. 5).**

Determination of MX gene

The HEP-2 cell line was subjected to total RNA extraction after treatment with non-toxic concentration E.A extract and IFN-α2a, that stimulate the induction of MX-mRNA as a marker of E.A extract and interferon antiviral activity. Detection of the MX gene was confirmed using specific primers in rt-RT-PCR. Specificity of this reaction was confirmed using negative control sample (non-interferon treated cells). Results indicated that the MXgene was expressed in the HEP-2 cell line induced by E.A extract and IFN-α2a while, no-enhancement of MXgene in the non- treated cells (negative control) **(Fig. 6).**

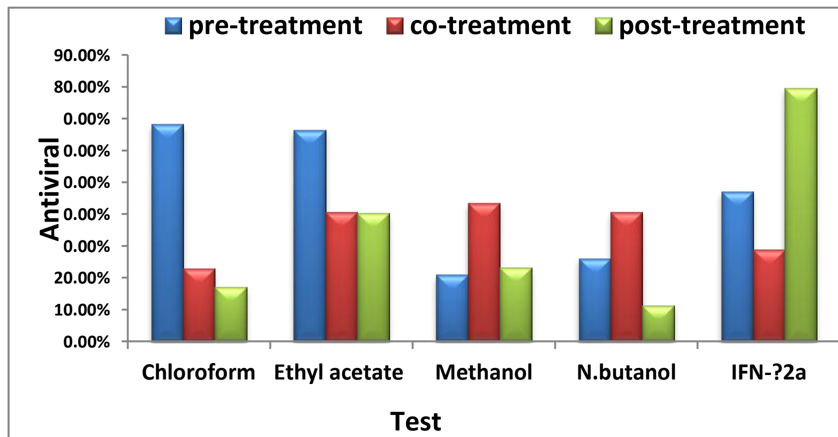

Fig. 5. Effect of safe concentration extracts compared with interferon on the viral activity.

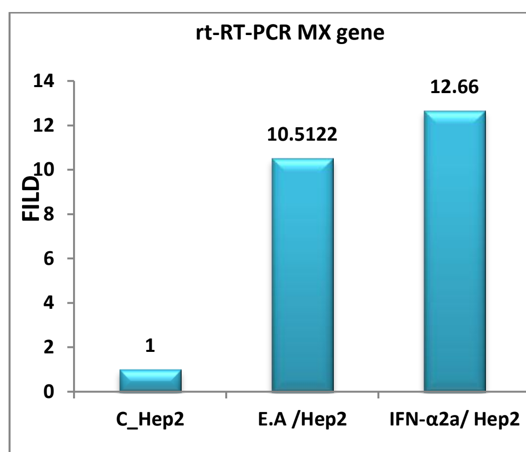

Fig. 6. rt- RT-PCR of MX gene of cell
C-Hep2: Control Hep2 cell
E.A: Ethyl acetate extracts of Moringa
IFN-α2a: Interferon Alpha 2a

DISCUSSION

There are some issues with the available antiviral drugs such as drug resistance; also toxicity could be a problem. So the trend was to use the herbal medicine as anti-viral reduce the side effects of antiviral drugs **(Shami, 2016).**

*Moringa*oleifera (MO) is a deciduous plant widely cultivated throughout tropical and subtropical countries of the world including Africa and Asia **(Hamad et al 2016 and Shunmugam, 2017).** *Moringa* is rich in phytochemical compounds that confer on the plant significant medicinal properties that could be valuable for treating certain ailments. The leaves of M. oleifera is a source of protein, iron, calcium, ascorbic acid vitamin A and antioxidant compounds such as carotenoids, flavonoids, alkaloids, vitamin E and phenolic compounds. Thus, its leaves could be used as an antiviral **(Mohamed, 2015 and Abd El-Hack et al 2018).**

In this study, different solvents have been utilized to obtain extracts from leave of *Moringa*. Effectively extract polar and non-polar were used to obtain the bioactive gradients **(Jung et al 2015).**

These extracts were analyzed by spectrophotometry for phenoliccontent, the highest concentration of phenol was in Ehyl acetate extract followed by n.butanol extract then chloroform then methanol and this is consistent with **(Younus et al 2015).**

Four extracts at different concentrations were applied to cytotoxicity and viability on HEP2 cell lineusing MTT assay. It was noticed that cytotoxicity induced was dose dependent. Cell viability inhibited, regarding the untreated control, it showed normal cell behaviors and morphology. These results showed that, the increase of polyphenols conc. was highly toxicity on HEP2 cell different studies had reported the *Moringa*oleifera contains phenolic compounds which possess cytotoxic activity at higher concentration on BHK-21cell **(Younus et al 2015)**.

Several studies had conducted to evaluate plant extracts against COXV. Evaluation of the antiviral activities differs from study to another **(El-Awady et al 2014).** In this study we used assay virus titer log reduction method by calculating the difference between virus titer of the control virus and its titer when it is used to infect pre-treated, co-treated and post-treated cells with the extract. the 4 extracts CL, E.A, M, and n.b of *Moringa* have shown remarkable antiviral activities when compared to interferon as positive control and further evaluation was done by evaluation of the virus loss using tissue culture titration technique In both techniques 24 hrs pre-treatment, co-treated and post-treated of the cells by the extract prior to the infection which shows if the plant is able to protect the cells against infection **(Ruffa et al 2004)** the prophylactic action involves interferon activity from the cells which can be induced by viral infection. Furthermore comparative evaluation was processed to measure the average loss of the virus titer as percentage loss (% depletion rate) which reflecting the antiviral potential of plant extracts on Hep2 treated cells compared to only virus treated cells. There were agreement with previous report **of El-Awady et al (2014) and Nasr-Eldin et al (2017).**

MXproteins belong to a family of ubiquitous proteins with antiviral potential induced in mammalian cells by alpha interferon, by double-stranded RNA,and by some viruses **(Chieux et al 2001).** Transfected HEP2 cells expressing MX allowed the demonstration that MX, an IFN type I-induced molecule, is a powerful antiviral protein able to inhibit diverse groups of viruses **(Chieux et al 2001).**So, MX gene was determined in HEP2 cells treated with E.A extracts, HEP2 cells treated with IFN -α2a, and the results showed highest level of MX gene transcription in HEP2 cells treated with IFN -α2a then the HEP2 cells treated with E.A extract compared to control cell and this agrees with **Chieux et al (2001) and Fahmy and Shoman, (2014).**

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مستخلصات المورينجا الغنيه بالبولي فينول كمضاد لكوكساكي بي فيرس

[19]

نجلاء ابوزيد سيف[1]-.بدوي عبدالسلام عثمان[2]– علي فهمي محمد[3]– خالد عبدالفتاح الدجدج[2]

1. الهيئة العامة للتأمين الصحي – مستشفى صيدناوي – القاهرة– مصر
2. قسم الميكروبيولوجيا الزراعية– كلية الزراعة– جامعة عين شمس– ص.ب 68– حدائق شبرا 11241– القاهرة– مصر
3. الشركه القابضه لانتاج الامصال واللقاحات (فاكسيرا)،الجيزه – مصر

*Corresponding author: naglaa_Abozied@yahoo.com

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الموجـــــــز

فيروس الكوكساكي بي (COX–BV)، هو من أهم الفيروسات المنقوله بالغذاء والتي تسبب امراض عديده للانسان منها مرض السكر المعتمد على الأنسولين، التهاب عضله القلب، الالتهاب السحائي، الالتهاب الكبدي.

المورينجا نبات غني بالمواد الكيميائية مثل البروتين والحديد والكالسيوم وحامض الأسكوربيك فيتامين A ومركبات مضادة للأكسدة مثل الكاروتينات ، الفلافونويد، القلويدات، وفيتامين E والمركبات الفينولية، لذلك فهي مفيدة في الناحية الطبية والتغذية، وتم استخدامها في الآونة الأخيرة كمضاد للفيروسات.

في هذه الدراسه تم استخلاص المواد الكيميائيه النباتيه من اوراق المورينجا بواسطه اربع مذيبات مختلفه كلوروفورم (CL)، ايثيل اسيتات (E.A)، ميثانول 80 % (M) و ن. بيوتانول (N.b). تم قياس تركيز الفينول في الاربع مستخلصات باستخدام جهاز الاسبكتروفوتوميتر وكانت النتائج كالاتي: وجد أن مستخلص E.A كان أعلى تركيزا (26.60 مجم / مل) متبوعاً بمستخلص N.b(21.68 مجم / مل) ، ثم مستخلص CL (15.38 مجم / مل) ثم مستخلص M (14.22 مجم / مل). تم دراسه التأثير السام للاربعه مستخلصات بتركيزات مختلفة علي خلايا HEP2 باستخدام اختبار MTT، أشارت النتائج إلي أن تركيز 100 ميكروغرام / مل للأربعة مستخلصات كانت آمنة بشكل كبير للخلايا HEP2. وكان بقاء الخلية بنسبه 91.33 و 93.19 و 92.29 و93.17 في المائة لكل من CL و E. و M وN.b على التوالي وكانت قيمة IC50 للمستخلصات CL و E.A و M أقل بكثير من مستخلص N.b.

كما تم تقييم تأثيرالأربعة مستخلصات لأوراق نبات المورينجا كمضادات لفيرس الكوكساكي بـــي (COX–Bv) والتأثيرات المقابلة علي حيوية خلايا HEP2 بثلاث طرق قبل المعالجة (pre-treatment) والمعالجة المشتركة (co-treatment) وما بعد المعالجة (post-treatment). وكانت النتائج كالاتي:- في المعامله pre-treatment، أظهرت النتائج ان أعلي نسبه انخفاض في تركيزالفيروس (في العدوى بالفيروس) كان في مستخلص CL بنسبه (68.3%) يليه مستخلص E.A بنسبه (66.4%) يليه مستخلص Nb (26.1%) يليه مستخلص M (% 21) مقارنة بالانترفيرون الفا (IFNᾰ-2a) الذي خفض تركيز الفيروس بنسبه (47.2%) ومن ناحية أخرى، في حاله المعامله post-treatment، أظهرت النتائج ان الاربعه مستخلصات ,Nb, M, EA, CL

تحكيم: ا.د فوزي المرسي أبو العباس
ا.د سحر أحمد حافظ شومان

سجلت اقل نسبه انخفاض في تركيز الفيروس (17.1 ٪) و(40.3%) ,(23.2%),(11.3%) علي التوالي مقارنه بالانترفيرون الفا (IFNᾰ-2a) الذي خفض تركيز الفيروس بنسبه (79.7%) وهذا يعني ان مستخلص EA أعطي اعلي انخفاض في تركيز الفيرس. اما بالنسبه للمعامله co-treatment فقد وجد أن المستخلصات Nb, M, EA اظهرت اعلي انخفاض في تركيز الفيرس (40.7%) و(43.6%) و(40.7%) علي التوالي مقارنة بالانترفيرون الفا (IFNᾰ-2a) الذي خفض تركيز الفيرس بنسبه (28.9)٪ في حين أن مستخلص CL خفض تركيز الفيرس بنسبه اقل من الانترفيرون الفا (22.9%).

كما تم رصد مستوى التعبير الجيني MX في خلايا Hep2 كمقارنه وخلايا Hep2 المعامله بمستخلص الايثيل اسيتات Hep2-EA وخلايا Hep2 المعامله بالانترفيرون الفا Hep2-IFNᾰ-2a وسجلت النتائج ارتفاع كبير في مستوي MX جين في Hep2-IFNᾰ-2a (12.66) يليه Hep2-EA (10.51) مقارنة مع مستوى MX جين الخلايا الكنترول Hep2 (1)

الكلمات الدالة: مستخلصات المورينجا، المركب الفينولي IC_{50}– النشاط المضاد للفيروس– جين Mx