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RESEARCH ARTICLE

ANTI-HEPATITIS C ACTIVITY OF EXTRACTED FRACTION OF PSEUDOMONAS OLEOVORANS USING HEPATITIS C VIRUS CELL CULTURE SYSTEM

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Abstract

One of the primary goals of antiviral research in recent decades has been the biosynthesis of new antiviral agents. The large number of bioactive agents extracted from microbial isolates led to the building of connections between products through antiviral screening. Using a genotype 4 plasmid, a microbial antiviral extract fraction from Pseudomonas oleovorans evaluated against HCV in Hepatitis C cell culture. The cytotoxicity assay using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] for determination of the ability of the mitochondrial enzyme succinate dehydrogenase to breakdown the tetrazolium salt and reflect cell viability indicates the inhibitory concentration of 50% of the fraction is732.2 µg/mlto be further applied for the concentration conventionalin the antiviral assay. The antiviral assay screenedthrough inhibition of viral entry, interference with viral replication, and blocking viral assembly and release assays. In both viral cell pre-treatment and viral replication interference assays, the extract inhibited viral infection in HCV cell culture systems with reduction in extracted HCV viral RNA from 9,837 to <12 and 4,035 to < 12 IU/ml respectively. On the other hand, there was a slight reduction in viral RNA in case of inhibiting assembly and releasemechanism from 4950 to 3710 IU/ml after detecting RNA using Real-time PCR.

Introduction:-

The infection with the hepatitis C virus (HCV) results in a viral hepatitis C whichis called the "silent killer," due to the long incubation period of the virus and the disease develops into a chronic phase. Chronic HCV infection is determined according to the effect on the liver degree of damage while remaining untreated from infection. The liver cycle after infection with HCV, is fibrosis, cirrhosis, and even liver cancer [1]. The correlation between HCV and other diseases also introduces the need for developing antiviral agents. Cryoglobulinemia, an endothelial injury results from the precipitation of patients' serum or plasma and end organ damage associated with HCV infection [2]. Also, arthritis and arthralgia are most frequent, to the presence of cryoglobulinemia that may impact vasculitis and sicca syndrome [3]. Diabetes (DM) is also one of the strongly related disorders to liver disease either, hepatogenous diabetes or (HD) or liver disease arisingluckily with DM [4,5].The importance of studying HCV has important impact on both hepatic and non- hepatic related disorders [6].

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Replication of HCV and eradication of infection detected in a large number of patients with choric hepatitis C infection through using HCV protease inhibitor and other antiviral agents with anti HCV activity (such as peg-interferon, ribavirin, and the direct-acting agents (DAAs) blocking the NS5A and NS5B viral regions in a high quantity of HCV patients. At least four boceprevir, glecaprevir, grazoprevir, paritaprevir, simeprevir, and telaprevir are examples of HCV protease inhibitors taken the approval for use in the United States. In addition to asunaprevir, danoprevir, faldaprevir, sovaprevir, and vedroprevir have been under evaluation in clinical trials. Protease inhibitors which are approved against the common Genotype 1 spreading widely in the USA, and are further to be used in other HCV genotypes [7].

The structural proteins of HCV, which has been as active antiviral targets in viral particle, including the core protein and the envelope glycoproteins E1 and E2. The nonstructural proteins including the p7 viroporin, the NS2 protease, the NS3-4A complex harboring protease and NTPase/RNA helicase activities, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase [8].

The search for new antiviral agents is required to widen the ability of antiviral to obtain the balance between strong activity and limited side effects. The use of herbal plants over centuries against different diseases including viral diseases reflected the image of other natural and microbial metabolites as antiviral agents. Glycyrrhizin (GL), extracted from Glycyrrhizaglabra, a perennial herb, demonstrated anti-Hepatitis C activity with the reduction of 50% of viral titer [9]. Red and blue-green algae reported to contain the proteins Griffiths in (GRFT) and scytovirin (SVN) with anti-HCV activity [10]. Vitamin D detected as a natural antiviral mediator through the determination of a synergistic effect in the inhibition of HCV viral production in the combination of vitamin D3 or calcitriol and interferon [11]. Agrimoniapilosa (AP), Gallarhois (RG), and their mixed compound also have anti-HCV by strongly inhibiting the expression of viral proteins from distinguishing the expression level of HCV Core 1b and NS5A [12]. Anti-HCV compounds three-2,3-bis (4-hydroxy-3-methoxyphenyl)-3-butoxypropan-1-ol and medioresinol (, which were extracted from Crataegus cuneate, extracted from crude drugs from herbal plants significantly inhibited HCV production in a dose-dependent manner [13]. The inhibition of HCV replication detected by using the compounds APS a natural alkaloid extracted from Maytrenusilicifolia, and the lignans from Peperomiablanda through decreasing luciferase activity and expression of HCV protein [14]. A natural compound Tylophorine, derived from Tylophoraindica plants, led to the discovery of 5-Oxo-1-[(2,3,6,7-tetramethoxy-9-phenanthrenyl) methyl]-L-proline (O859585) and 2,3,6,7-tetramethoxy-9-phenanthrenecarboxylic acid (T298875), exhibited anti-HCV activity by blocking attachment ofHCV and neutralizion of free viral particles without interfering with other stages of the viral life cycle and stimulation ofinterferon [15]. CarapaProcera DC. and Pericopsislaxiflora (Benth. ex Baker) Meeuwen crude extracts showed anti-HCV and found to be rich in polyphenols, including tannins such as procyanidins A2 [16]. Also triterpenes, naringenin, Proanthocyanidin, curcumin, Epigallocatechin-3-gallate, quercetin and abrogates developedvaried anti-HCV activity [17].The anti-HCV activity of Lentinula edodes mycelia solid culture extract due to the active low-molecular-weight lignin, which inhibited HCV particles' entry in cell culture (HCVcc)[18].

A pyrazine derivative, trypilepyrazinol, and another ergostane analog, 3β-hydroxyergosta-8,14,24(28)-trien-7-one were isolated from the marine-derived fungus Penicillium sp. IMB17-046. Both compounds demonstrated anti-HCV activity [19]. Raistrickindole A were isolated from the marine-derived fungus Penicilliumraistrickii IMB17-034 showed inhibitory activities against the hepatitis C virus [20]. A group of prenylated p-terphenylquinones extracted from the plant pathogenic fungus Cytospora sp. strain CCTU A309 exhibited antiviral activity against hepatitis C virus (HCV) [21]. The endophytic fungal species, Diaportherudis MERVA25, Penicilliumpolonicum MERVA43P, Lophiostoma sp. MERVA36, Auxarthronalboluteum MERVA32, Trichodermaharzianum MERVA44, Fusariumoxysporum MERVA39, Aspergillus versicolor MERVA29, and Penicilliumchrysogenum MERVA42 extracts, obtained from the marine sponge, Hyrtios erectus showed significant hepatitis C virus (HCV) inhibition [22]. Marine endophytic Streptomyces strains Streptomyces sp. MORSY 17,Streptomyces sp. MORSY 22, Streptomyces sp. MORSY 25, Streptomyces sp. MORSY 36, Streptomyces sp. MORSY 45, and Streptomyces sp. MORSY 50isolated from marine soft coral Sarcophytonconvolutum exhibited wide spectrum antiviral against the hepatitis C virus [23].

Material and Methods:-

Bacterial extraction and fractionation:

Pseudomonas Oleovorans was cultured in Casamino acids Yeat extract Glucose)(CYG) broth medium composed ofCasamino acids, 5 g; yeast extract, 0.5 g; glucose, 1 g; NaCl, 6.8 g; KCl, 0.4 g; MgSO4.7H2O, 0.2 g; CaCl2

(anhydrous), 0.2 g;1,000 ml of distilled water and pH 7.2. The culture supernatant was extracted using ethyl acetate and further fractionated using methanol (90%) and n-hexane and the selected fraction was subjected to this analysis of anti-HCV using HCV cell culture system [24].

Cell line

Huh7.5 a gift from professor ChalresMoen Rice, Rockefeller University, NY, USA were seeded using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% penicillin-streptomycin, 1% non-essential amino acids, and 10% fetal bovine serum (FBS) in a humidified cell incubator containing 5% CO2 and 95% air at 37°C.

Plasmid

The plasmid for transfection and infection 4a (ED43/C-NS2/NS5A) in the study was kindly supported by the Department of Infectious Diseases and Clinical Research Centre, University of Copenhagen, Denmark.

Cytotoxicity of extracted fraction using MTT assay

MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was used for confirmation of the viability of Huh-7.5 cells. For the determination of the non-cytotoxic concentration of the analysis of Pseudomonas Oleovorans's fraction, liver cells were seeded at a density of 5 × 105 in 96 well plate. Untreated cells were considered as control and added different concentrations of the fraction (1000, 500, 250, 125, and 62.5 µg/ml) were to the 24 hours previously cultured cells. After 48 h the cells of incubated at 5% CO2, MTT was added then the plate was incubated for 3h at 37 ◦C. For color development, 100 µl of DMSO was added to each well and mixed thoroughly. Then the optical density was measured at 570 nm. The determination of viable cells and percent of viability was calculated from the equation Cell Viability %= absorbance of treated cells / absorbance of controlled cells ×100. The percent of viable cells and drug concentration was plotted to find the 50% inhibitory concentration (IC50), the concentration required to cause toxic effects in 50% of adherent cells, which was valued from graphic plots using GraphPad Prism software (San Diego, CA, USA) [25].

Anti-HCV analysis of extracted Pseudomonas Oleovorans's fraction on Huh-7.5 cells

In vitro transcription was carried out using plasmid containing approximately 40 µg/mL.Following transcription steps, the final concentration of synthesized RNA was determined using Nanodrop. Huh7.5 cells were seeded and incubated for 24 h, and then cells were transfected with HCV RNA. After incubation for 48 h culture filtrates were collected every 2 days over 7 days and concentrated using Amicon ultra 15 centrifugal filter units at -4°C for 30 minutes. Following virus concentration, optimization experiments were performed using real-time PCR to reach maximum infection outcome according to the concertation of the virus [26].

Three different approaches illustrated in Fig.1,were used to determine the extract anti-HCV activity according to the antiviral targets. Cells were seeded at an exponential growth phase of 35 × 104 cells per well into a 6-well plate for 24h to allow the formation of an approximately 90% cell monolayer. RNAs diluted in a serum-free medium to the optimum concentrations required for infection. As the first approach is the evaluation of the extract for inhibiting viral entry, the extract fraction dissolved in DMSO with a concentration less than the IC50 was added to the virus and incubated at 37 °C with 5% CO_2 for 4 hoursthen applied on cell monolayers to introduce infection. Another non-treated viral sample is set as a control. The second approach is to determine the ability of the extract to inhibit viral replication, so another sample of the virus was introduced to the cells monolayer to induce infection then after 4 hours of incubation; the extract subtoxic concentration was added to the cells. A control sample with free media was used as a positive control of infected HCV cells. The last approach is studying the ability of the extract to decrease viral assembly and release. The same concentration of the extract was mixed with the virus and introduced to cells, then incubated for 4 hours. The virus control sample was used using free media to determine the difference between treated and untreated cells. After required incubation for each well, complete media DMEM supplemented with 1% penicillin-streptomycin, 1% non-essential amino acids, and 10% fetal bovine serum (FBS) was added to complete the volume to 2 ml of each well. All samples were incubated at 37 °C in the incubator with 5% CO2 for 24 hours then themedia was replaced to complete the incubation for a further 24 hours [27].

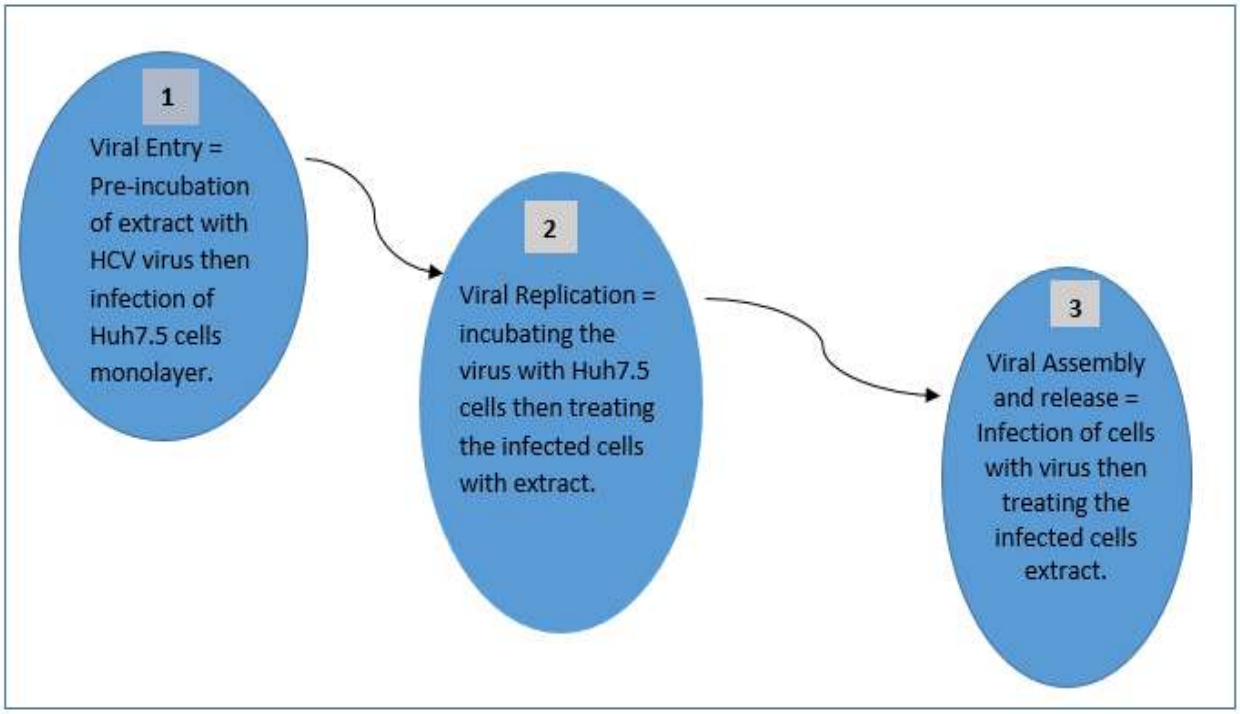

Fig. 1:- Antiviral screening through different modes of action.

Viral Load Analysis by Real-Time PCR:

The RNA copy number of hepatitis C virus (HCV) in Huh7.5 cells was determined by using HCV quantitative Real-Time PCR Kit (Qiagen, USA). After incubation, viral RNA was isolated from Huh7.5 cells using RNeasy Plus Mini Kit (Qiagen, Germany). HCV RNA load was determined using Qiagen HCV real-time PCR Kit (USA) according to the manufacturer's instructions. The real-time PCR program comprised 15 minutes at 50°C (for cDNA synthesis), 10 minutes at 95°C (for initial denaturation) for 45 cycles, including denaturation of 95°C for 15 seconds, 20 seconds annealing at 55°C and an extension for 15 seconds at 72°C [28].

Results And Discussion:-

Cytotoxicity using MTT assay

MTT is a reliable, sensitive, and quantitative cell viability assay. The assay is based on the cellular mitochondrial dehydrogenase reduction capacity of yellow water-soluble substrate 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) in the living cells followed by the production of the insoluble dark blue/purple formazan product indicating the viable cell number. The result obtained showed that the extracted Pseudomonas oleovorans's fraction concentration of 1000 µg/ml was toxic to cells (Fig.2). The inhibitory concentration (IC50), the concentration required to cause toxic effects in 50% of adherent cells, was valued from graphic plots using GraphPad Prism software was 732.2 µg/ml [29].

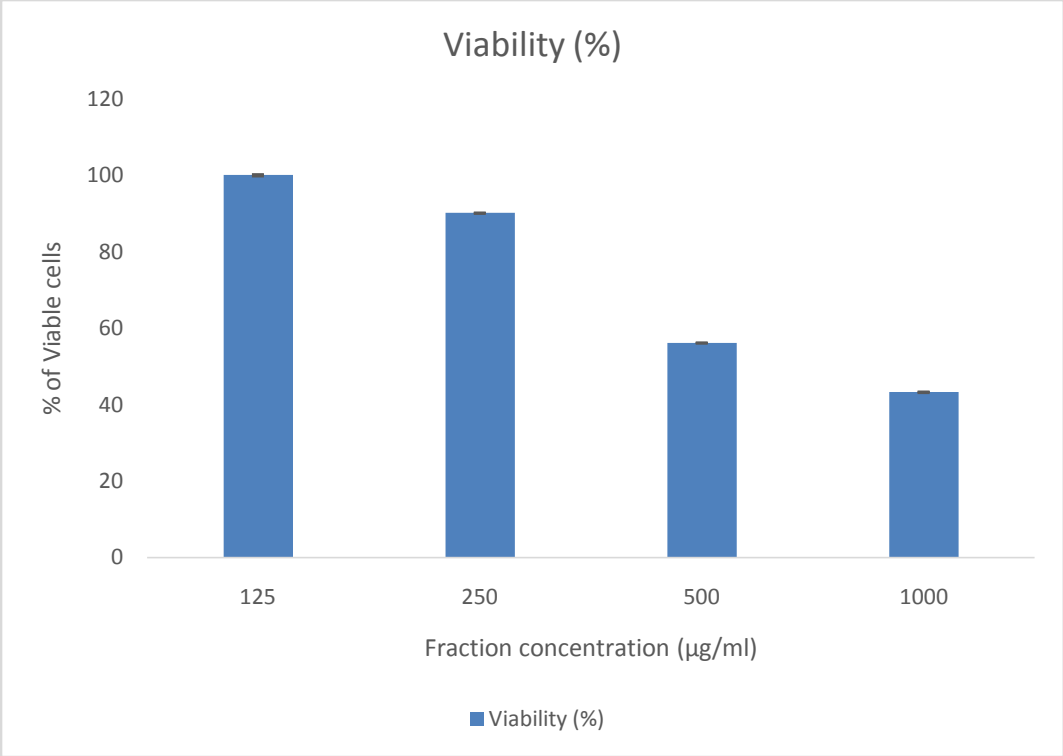

Fig. 2:- Viability of 7.5 cells after treatment with MTT.

There are three approaches to antiviral agents to be active and provide a great effect on the virus. The first one is through blocking of HCV entry, the second is interference with the HCV replication cycle and the last one is inhibition of assembly and release. HCV patient's sera with high viral load infecting the cells to test the ability of extract to inhibit viral entry and replication. The selected bacterial fraction 0.1 µg / ml containing tetradecanoic acid and hexadecanoic acid methyl esters was capable of inhibiting viral entry and replication [30].

A-Entry of the virus

The detection of viral load after treatment with bacterial fraction 500 µg/ml and virus in the same step resulted in complete inhibition of the virus as detected with RNA viral load using Real-time PCR from 9,837 to <12 IU/ml. The viral entry is a multistep process and through small molecules and compounds extracted from the natural sources, the visions thus block viral infectivity. The extracts inhibit infection of the virus by binding to HCV envelope glycoproteins E1 and E2 thus blocking the entry of virusinto human hepatocytes. Binding assay showed the reduction in attachment of HCV through pre-incubation of the extract resulted in decreasing the quantity of HCV RNA bound to the surface of cell. Some antivirals act on E1 and E2 glycoproteins causing conformational changes on the particles of the virus, thus disturbing viral particles and cell surface interaction [10,31].

B-Replication of the virus

Blocking the synthesis of new genomes of the virus is a successful strategy for Inhibiting HCV replication by new antivirals. The extract fraction 500 µg/ml showed an effect in the reduction of RNA extracted from cells after infection from 4,035 to < 12 IU/ml. The main target of this step is preventing the construction of HCV viral genomes in infected cells. Many extracts block genome replication through the reduction of both protein and RNA levels in infectious replicon systems. Inhibitory effect of extracts targeting both NS2 or NS3 protease and NS5A or NS3/4A inhibition, inactivation of phosphatidylinositol 3-kinase (PI3K)/AKT pathway affects the efficiency of virus replication and production [31].Another approach is the reduction of the levels of mature miRNA-generating complexes in HCV replication[32].

C-Assembly and release of the virus

The comparison between treated and untreated infected cells with extract in this assay showed a slight reduction as infected cells showed 4950 IU/ml after detecting RNA using Real-time PCR and 3710 IU/ml in treated cells with the

extract. The decrease in extracellular HCV positive-strand RNA demonstrating a decrease in HCV core secretion reflects the effect of antiviral extracts on blocking the assembly of intracellular HCV particles [33]. The triggering of PPARα which is a transcription factor associated with lipid metabolism in the liver is connected to the decrease of lipogenesis and VLDL secretion [34].

Effective antiviral targets depend on initiating, spreading, and maintaining infection, viral entry is depending on interfering with HCV viral entry. Antivirals may cause the blocking of virus–target cell interaction throughout attachment and binding, resulting in interference with post-binding events and fusionof the virus [35].

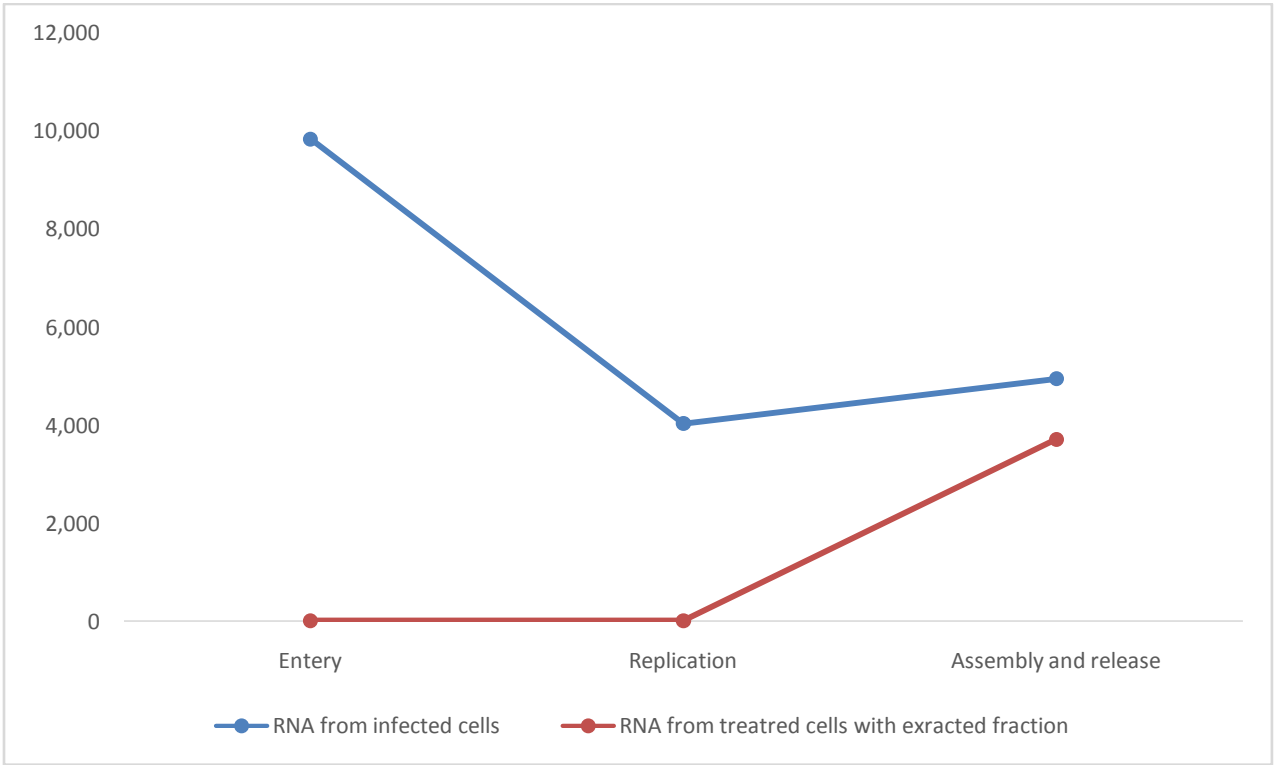

Fig. 3:- Anti-HCV of an extracted fraction of Pseudomonas oleovorans using viral entry, replication, assembly and release assays.

Fatty acid methyl esters extracted from natural resources showed a wide antifungal and antibacterial activity such as Sesuviumportulacastrum leaves [36], and biological activity has been more broad spectrum to anticancer in Peperomiapellucida leaf extract [37]. The hexadecanoic acid methyl ester was extracted from Gossypium seeds and evaluated with activity as antibacterial higher than an antifungal agent [38]. Hexadecanoic acid methyl esterin Vernoniaamygdalinamethanol fraction was describedas inhibitingthe growth and induce apoptosis in human gastric cancer cells[39]. FAME extract of the halophytic plant, Salicornia brachiata showed high antibacterial and antifungal activities [30]. Fatty acid synthesis also directed to the biological activity of being antibacterial [41]. Spirulina platensis n-hexane extract with antiviral and antimicrobial activity were detected with the presence of saturated, unsaturated fatty acids and monoterpene hydrocarbons. The antiviral activity of the total methanol extract based on the correlation between the synergistic effect of carbohydrates and phenolic compounds [42]. Extracts from brown and red algae from the black sea showed a remarkable inhibition in the replication of influenza and Herpes simplex viruses in cell cultures. The extracts analysis showed the presence of volatile compounds, some phenols, free fatty acids and their oxidized derivatives, eucalyptol, the methyl ester of phenylacetic acid, and 1,2-dihydroxy ethane sulfonate [43].

Conclusion:-

The evaluation of anti-HCV fraction using three different mechanisms showed the ability of the extracted fraction to develop antiviral activity against HCV through either interfering with vial entry or inhibiting viral replication in infected cells. In addition to entry and replication process, upon screening viral assembly and release process

reduced inhibition level detected. The results influenced in reflecting the impact of the fraction activityin targeting viral life cycle during attachment and replication.The development of new antiviral agents through biosynthetic pathways such as microbial metabolites could introduce new targets for viral development and treatment.

Conflicts of interest

There are no conflicts of interest to declare.

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