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Inhibitory Activities of Methanolic Extracts of *Carica papaya*, *Mentha piperita* and *Citrullus colocynthis* Against Dengue Viruses-2 viability

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ABSTRACT: This study targets the methanolic extracts from the leaves of *Carica papaya* (Papaya) and *Mentha piperita* (Mint), as well as the leaves and fruit extracts of *Citrullus colocynthis* (Tumma plant) for their antiviral activity against dengue virus type 2 (DENV-2) *in vitro*, utilizing the HepG2 cell line. The antiviral activity of the plant extracts mentioned above was assessed through a plaque formation assay on the HepG2 cell line. The methanolic extracts from the leaves of *C. papaya* and *M. piperita* demonstrated lower toxicity than the fruit extract of *C. colocynthis*. Notably, the fruit extract of *C. colocynthis* maintained the normal morphology of DENV-2-infected HepG2 cells with minimal cell mortality. The cell survival rates were found to be 80-100% for 0.30mg/ml and 0.20mg/ml of *C. papaya* and *M. piperita* leaves extracts and 0.08mg/ml of *C. colocynthis* fruit extract. The most effective inhibition of DENV-2 was attributed to the fruit extract of *C. colocynthis*, exhibiting 46.55% inhibition at 0.08mg/ml. In comparison, *M. Piperita* and *C. papaya* leaf extracts demonstrated 24.14% and 23.33% inhibition against 0.20mg/ml and 0.30mg/ml, respectively. The plaque forming unit (PFU/ml) at the highest concentration ensuring 100% cell survival was calculated as 1.5×10^6 , 1.4×10^6 , and 1.0×10^6 PFU/ml for *C. papaya* and *M. piperita* leaves extracts and *C. colocynthis* fruit extract, respectively. The study infers that the methanolic fruit extract of *C. colocynthis* exhibits a high potential for inhibiting DENV-2 compared to the other two extracts.

Keyword: *Carica papaya*, *Menthapiperita*, *Citrulluscolocynthis*, DENV2

INTRODUCTION

Dengue virus belongs to the Flaviviridae family and has four different serotypes (DENV 1- 4) and has been recorded in Southeast Asia, America, Africa, China, the Western Pacific, and most recently Europe (WHO, 2015). Dengue fever stands as the most prevalent arboviral disease in humans, exerting a considerable impact on public health (Correa et al. 2019). Dengue fever has become four times more common worldwide. Dengue fever has been four times more common worldwide in recent years. Annually, a substantial number of dengue cases are reported in tropical and subtropical regions worldwide. Approximately 500,000 individuals with severe dengue require hospitalization each year, with a notable proportion being children. The annual mortality rate for those affected is around 2.5 percent (WHO, 2015).

Aedes mosquito species have been identified as DENV vectors, with *Aedes aegypti* being the most effective in urban areas and *Aedes albopictus* less effective in causing large-scale epidemics (Lwande, 2020). *Aedes aegypti* is a peridomestic mosquito that breeds near the human population. It lays eggs in water containers and feeds on human blood several times

during its gonotrophic period. In the forests of West Africa and Malaysia, nonhuman primates were discovered to be infected with sylvatic DENV. Various *Aedes* mosquito species, such as *Aedes furcifer* and *Aedes luteocephalus*, transmit these viruses between primate hosts and are genetically distinct from the DENV found in urban cycles (Powell, 2018).

While it seems that all Dengue Virus (DENV) serotypes originated in primates, with each serotype independently crossing over to humans, the precise origin of DENV remains elusive. Evolutionary analysis suggests that the four DENV serotypes emerged approximately 1000 years ago, with significant human epidemics only manifesting in the last few centuries. The more extreme type of DHF/DSS started to appear in Southeast Asia, the Americas, and other tropical regions after World War II. Currently, there is no effective dengue vaccine or antiviral therapy. Dengue patients undergo a specific clinical trial before they recover without experiencing any side effects. Several studies have shown that the severity of a severe dengue attack is related to the extent of viral infection. As a result, antiviral drugs can help to minimize viral infection. Various studies have reported that extracts from

different plants have more significant antiviral effects than allopathic medications (Tang et al., 2012). Quinine, derived from the cinchona tree's bark, was used to treat malaria. Many diseases in the subcontinent are likely to be treated with ethno-medicinal or herbal treatments. Many people in Pakistan have historically used native medicine expertise related to herbal medicine (Prasad et al., 2009).

Antiviral properties have been identified for flavonoids, terpenoids, polyphenols, Tannins, baicalin, coumarins, phenolic alcohol, alkaloids, and glycosides (Tang et al., 2012; Zandi et al., 2012; Zandi et al., 2011). Flavonoids derived from plant leaf extracts, such as Neem, have potent antiviral properties that are also selective against dengue viruses (Zandi et al., 2011; Sairam et al., 2002). Quercetin is an antiviral agent in onions, citrus fruits, green and black tea, apples, tomatoes, and a few other plants (Ferrerres et al., 2010; Zhang et al., 2010).

Dengue fever has been subjected to treatment using a leaf extract from *C. papaya*; however, no definitive evidence of dengue inhibition has been established (Ahmad et al., 2011). The antiviral activity of soybean plants containing certain iso-flavone has also been confirmed against influenza

viruses (Liu et al., 2008). Alkaloids, methyl carpine, dehydrocarpaines, pseudocarpaine, flavonoids, benzyl glucosinolate, tannins, and saponins are some phytochemical constituents contained in *C. papaya* in previous studies (Oloyede, 2005). Peppermint leaf extracts have also shown antiviral activity against HIV-1 in T-cell lines (Geuenich et al., 2008). In vitro, the antiviral activity of papaya and peppermint leaf extracts against DENV viruses has not been published. Because of their low toxicity and high convenience in nature, plant extracts containing compounds and their derivatives are essential for the discovery and production of antiviral drugs (Kumar et al., 2012). *C. colocynthis*, also known as bitter apple, is used as a medicine in many countries. Fruits, nuts, roots, and leaf extracts are used as herbal medicine to treat inflammation, fungal, and bacterial infections, and diabetes (Kumar et al., 2008). Several chemical compounds derived from plants have been observed to inhibit virus replication. These compounds may be used as a natural remedy to control viral infection (Abiri et al., 2021).

There is currently insufficient information on the anti-dengue activities of medicinal plants such as *C. papaya*,

M. piperita, and *C. colocynthis*'s extracts. During this research, we tested the effects of leaf and fruit extracts on DENV-2 replication in the HepG2 cell line. The impact of each plant extract on DENV-2 replication, including virus adsorption, and direct virucidal activities, was assessed.

MATERIALS AND METHODS

Collection of Medicinal Plant Samples

Methanolic extracts of three medicinal plants *C. papaya*, *M. piperata* leaves, and *C. colocynthis* fruit were used in the study. Leaves of experimental plants were collected from Botanical garden GC University Lahore (N 31° 33' 23.65 and E 74°19' 41.952). And *C. colocynthis* fruit were collected from Layyah. Identification of plants was done by Dr. Sultan Herbarium, Department of Botany GC University Lahore with following voucher numbers *C. papaya* (GC.Herb.Bot.2236) leaves, *M. piperata* (GC.Herb.Bot.2885) leaves, and *C. colocynthis* (GC.Herb.Bot.2886) fruit. (Figure 3 a, b, c) Plant leaf extracts were prepared by following the published protocol (Luka et al., 2011) with some modifications. Fruit extract of *C. colocynthis* was prepared according to the method with some modifications described by (Emeruwa et al., 1982).

HEPG2 Cell Line Culturing

HepG2 cell lines were stored in cryovials within a liquid nitrogen cylinder and subsequently thawed to further culture. HepG2 cells were added along with Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, UK) with 10% Fetal Bovine Serum (FBS) (GIBCO, South America), in culturing Flasks. For proper propagation of cultured cells, some antibiotics like penicillin (100 units/mL) and streptomycin (100 µg/mL) (GIBCO, South America) were added to the culturing flask for safety. The cultures were incubated at 37 °C with 5% CO₂ and allowed to grow until reaching 70%-100%. Then washing of culturing flask has been done (to which the cells were attached) with normal saline. Splitting of cells was done and incubated with trypsin-EDTA until the cells fully detached from the surface of the culturing flask. Then culturing flask was observed inverted microscope to confirm detachment. After that, the mixture was centrifuged at 3000rpm for 5 minutes in a 15ml tube by adding FBS. After centrifugation, the supernatant was removed, and the pellet was re-platted (Maqbool et al., 2019)

Cytotoxicity Assay

Cytotoxicity of plant extracts was determined using a Neutral red (NR) uptake assay (Repetto et al., 2008). Briefly, 2300 cells/well were plated in 96 well plates in triplet for each treatment. After 24 hours, cells were proliferate to 10000 cells washed with pre-warmed PBS and 200 μ l of media with appropriate concentrations of each plant extract (Tables 1, 2, and 3) and incubated for 48 hours. Collection of Serum Sample for Virus Stock and Preparation of DENV-2 Stock Virus stock was taken from dengue patients. 1 ml of human sera positive for DENV-2 was mixed with DMEM medium and was slowly put on the hepatocytes grown in a monolayer in 25cm² culture flasks. After 4 days of inoculation, the cells were lysed, centrifuged, and viruses were harvested. This virus stock was used for plaque assays (Medina et al., 2012).

Viral Titration by Plaque

The antiviral activity of extracts was determined by plaque reduction assay in vitro performed following the VIRAPUR protocol. A cell suspension (with 1×10^6 cells/ml) was prepared in DMEM and seeded in a 6-well plate (2.5 ml of solution). Plates were incubated at 37 °C with 5% CO₂ for 24 hours. For viral dilution serial dilution of 20 μ l of viral samples was prepared using four 5

ml tubes similarly, 20 μ l of mix from first tube was added to the next. Process was repeated through all tubes. Each tubes containing 20 μ l of viral sample mixed with 2ml PBS containing 10^{-2} , 10^{-4} , 10^{-6} and 10^{-8} viral concentrations. After incubation, media was drawn out from wells and immediately added with 2 ml of agarose/media mixture. The plate could sit for 15 minutes until the agar overlay turned solid and incubated at 37 °C with 7.5-10% CO₂. Plaques were developed by the 5th day of incubation as white dots on the cell monolayer were visible. Plaques were visualized and counted under an inverted microscope. After counting plaques, the concentration of initial viral suspension (PFU/ml) was calculated using the following formula:

PFU/ml = average no. of plaques \times 1 / Dilution factor \times Volume of diluted virus added.

Plaque Reduction Assay

Plaque reduction assay was performed using a 24-well plate seeded with 1×10^6 cells/ml and incubated at 37 °C for 24 hours. When a confluent monolayer was obtained, 1 ml of media was aspirated from each well. 900 μ l of different extract concentrations in DMEM along with 100 μ l of 10^{-2} (10×10^{-4}) viral dilution were added in

triplicates and incubated at 37 °C for 4-16 hours. After an incubation period, half media was drawn out from wells and immediately added with 2 ml of molten agarose /media mixture. The plate was allowed to sit for 15 minutes until the agar overlay turned solid and incubated at 37 °C with 7.5-10% CO₂. Plaques were developed by the 5th day of incubation as white dots on a cell monolayer. Plaques were visualized and counted under an inverted microscope and PFU/ml was calculated as described above. Reduction in viral titer after extract treatment was determined by comparing PFU/ml with control (Zameer et al., 2005).

Statistical Analysis

Tested results were compared with their respective controls and statistically analyzed by way of ANOVA Tukey's Mean separation procedure test at a 95% confidence interval of the difference (SPSS Version 13.0 SPSS In).

RESULTS

Cytotoxicity of Medicinal Plants Extracts

The cytotoxic effects of *C. papaya*, *M. piperita* leaves extract, and *C. colocynthis* fruit extract were evaluated on the HepG2 cell line using the Neutral Red assay as outlined in the materials and methods section. In all three cases, the viability of HepG2 cells demonstrated an inverse relationship with the concentration of the respective extracts. The Maximum Nontoxic Dose (MNTD) for *C. papaya*, *M. piperita*, and *C. colocynthis* was determined to be 0.02 mg/ml, showing 100% cell survival. Notably, 80% cell survival was observed at concentrations of 0.4 mg/ml for *C. papaya*, 0.20 mg/ml for *M. piperita*, and 0.08 mg/ml for *C. colocynthis*. However, the minimum cell survival was recorded at 1.4 mg/ml for *C. papaya* and 0.8 mg/ml for *M. piperita* and *C. colocynthis* (refer to Fig. 1: a, b, c, d).

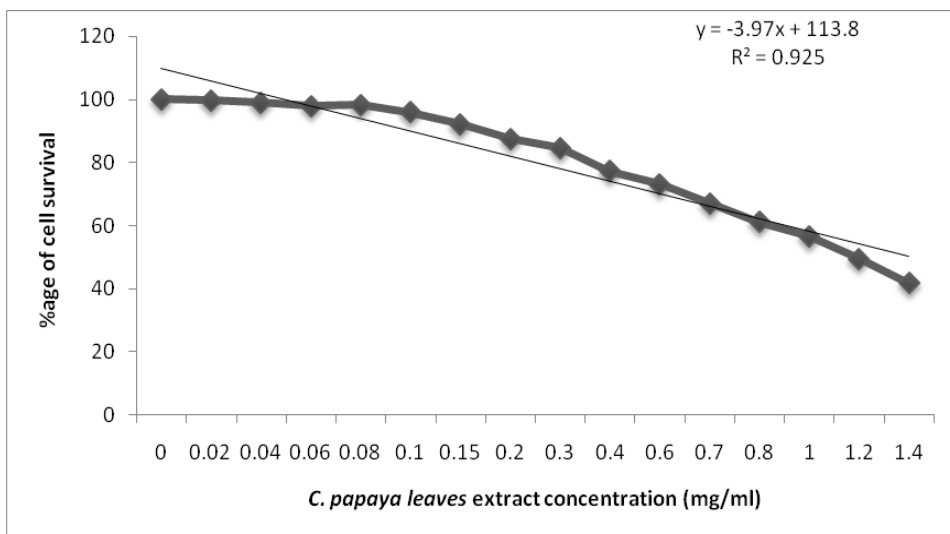


Figure 1: (a) Cytotoxicity of crude *C. papaya* leaves extract on HepG2 cell line.

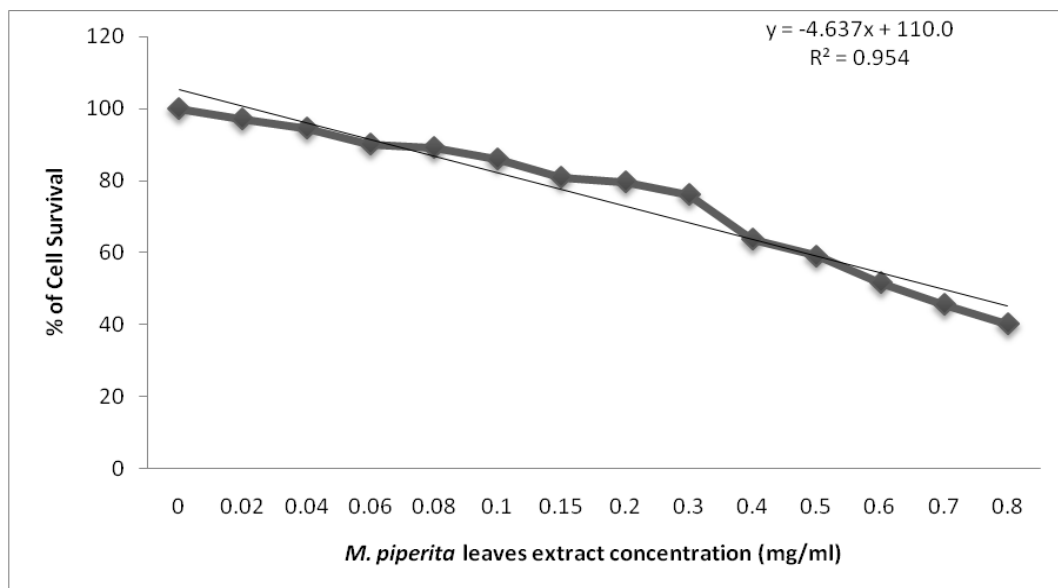


Fig. 1: (b) Cytotoxicity of crude *M. piperita* leaves extract on HepG2 cell line

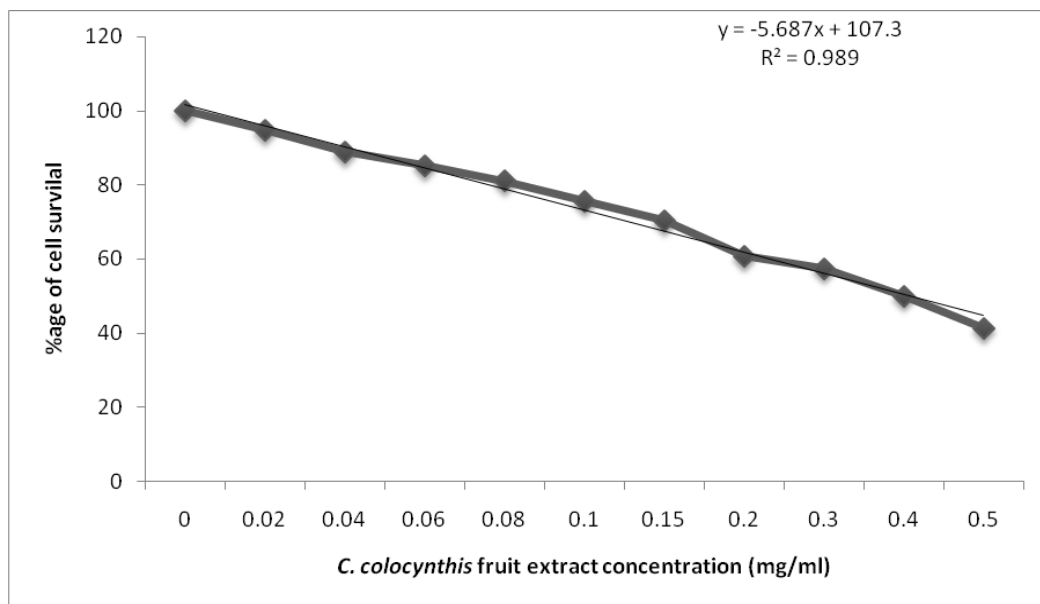


Fig. 1: (c) Cytotoxicity of crude *C. colocynthis* fruit extract on HepG2 cell line

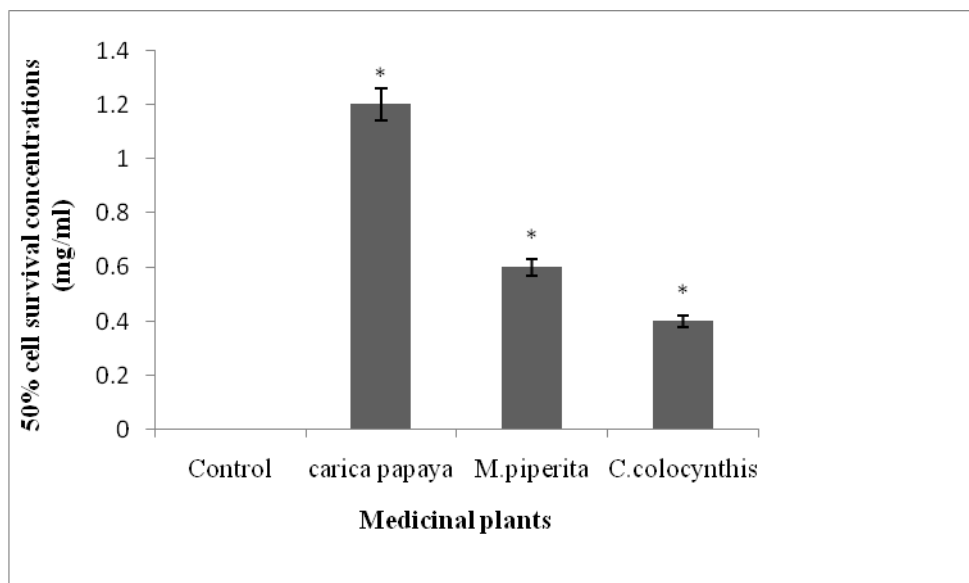


Fig. 1: (d) Comparative cytotoxic effect of extracts of *C. Papaya*, *M. piperita*, and *C. colocynthis* on HepG2 cell line.

* Represents treatment groups were highly significant ($p < 0.05$) with their respective controls at a 95% confidence interval. *C. papaya* ($p = 0.008$), *M. piperita* ($p = 0.001$), *C. colocynthis* ($p = 0.000$).

The Concentration causing 50% cell survival (CC50) for *C. papaya* was determined to be 1.20 mg/ml, for *M. piperita* it was 0.6 mg/ml, and for *C. colocynthis* it was 0.40 mg/ml.

colocynthis, it was 0.4 mg/ml. Importantly, no cytotoxicity was observed at a concentration of 1% DMSO, which was initially used for dissolving the crude plant extracts.

Top of Form

Antiviral Activity of Medicinal Plant Extracts.

The methanolic extracts of all mentioned plants were tested to determine their ability to inhibit viral particles after adsorption on the HepG2 cell line. The DENV-2 was inhibited by the methanolic extracts of *C. papaya*, *M.*

Piperita, and *C. colocynthis* with 23.3%, 24.14%, and 46.55% against concentrations of 0.3mg/ml, 0.2mg/ml and 0.08mg/ml concentration respectively. For *C. papaya* leaves extract, the number of plaque forming units (PFU) for 0.02mg/ml, 0.15mg/ml, and 0.30 mg/ml concentrations was 1.8×10^6 , 1.5×10^6 and 1.5×10^6 PFU/ml respectively. The result indicates a gradual reduction of plaques and inhibition of DENV2 by percentages of 10%, 21.67%, and 23.33% against the above concentrations respectively (Figure 2 (a)).

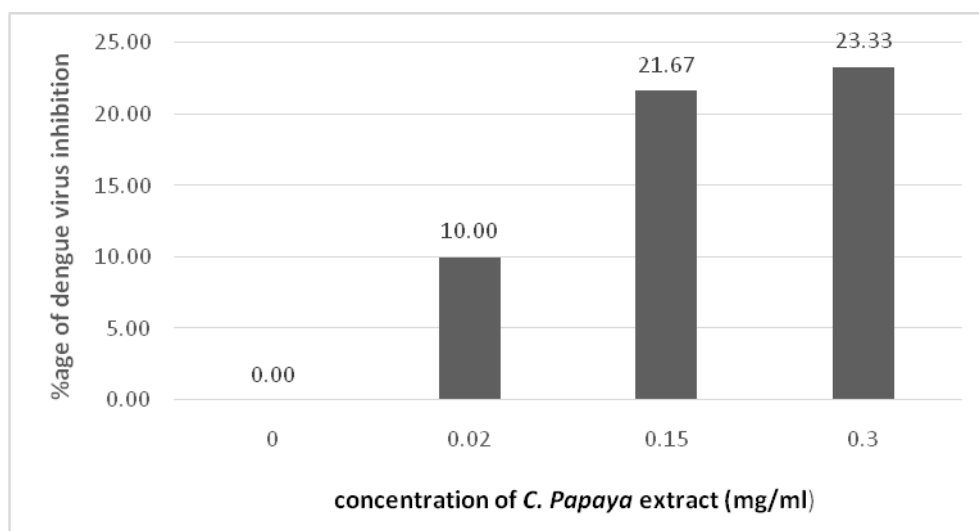


Figure 2: (a) Graphical representation of % age inhibition of DENV2 by methanolic extract of *C. Papaya* leaves in HepG2 cell line

.For *M. piperita* leaves extract, the number of PFU for 0.02 mg/ml, 0.06 mg/ml, and 0.20 mg/ml conc. was 1.8×10^6 , 1.6×10^6 and 1.4×10^6 PFU/ml, respectively. The result indicates a

gradual reduction of plaques showing inhibition of DENV2 by percentages of 6.90%, 13.79%, and 24.14% against the above concentrations, respectively (Figure 2b).

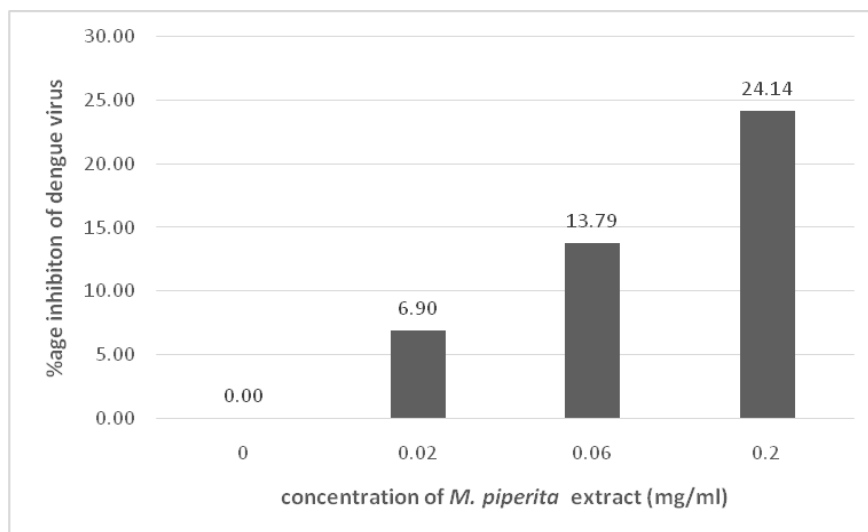


Fig. 2: (b) Graphical representation of % age inhibition of DENV2 by methanolic extract of *M. piperita* leaves in HepG2 cell line

For *C. colocynthis* fruit extract, the number of PFU for 0.02 mg/ml, 0.04 mg/ml, and 0.08 mg/ml concentrations was 1.6×10^6 , 1.3×10^6 , and 1.0×10^6 PFU/ml respectively. The result shows a

gradual reduction of plaques indicating inhibition of DENV2 by percentages of 17.24%, 31.3%, and 46.55% against the above concentrations, respectively (Figure 2c).

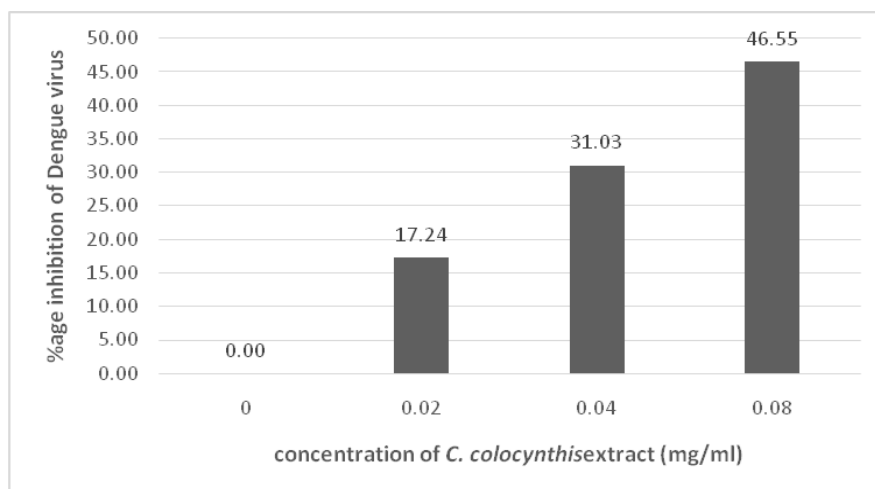


Fig. 2: (c) Graphical representation of % age inhibition of DENV2 by methanolic extract of *C. colocynthis* Fruit in HepG2 cell line

A comparison of % age inhibition by all three plants is shown in (Fig. 2d). The

means of control and all groups were analyzed, suggesting all results were significant.

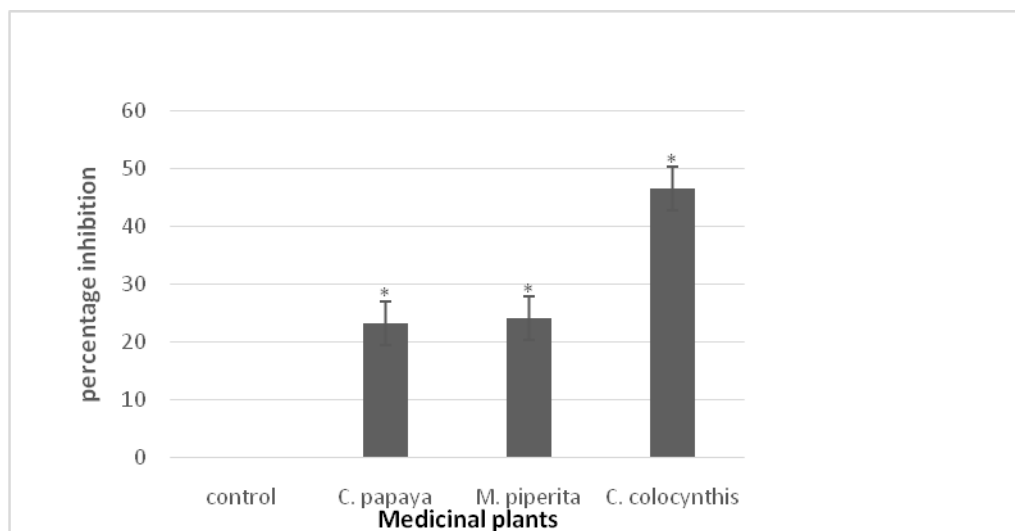


Fig. 2: (d) Comparison of % age inhibition of DENV-2 by methanolic extracts of three plants in the HepG2 cell line

* The mean differences between control and treated groups are significant at ($p < 0.05$) 95% confidence level. *C. papaya* ($p = 0.000$), *M. piperita* ($p = 0.002$), *C. colocynthis* ($p = 0.000$)

Discussion

There are currently no antiviral medications available for dengue patients, but they are treated with a variety of strategies such as fluids and blood plasma transfusions (Correa et al. 2019). The current study was carried out to screen medicinal plants with substantial antiviral activity against DENV-2, per WHO research priorities. a previous clinical trial, Yadav et al. (2017) recorded a substantial improvement in platelet count in dengue fever patients treated with *C. papaya* leaf extract. However, in the current

study, MNTD was the present culture at concentrations of 0.3, 0.2, and 0.08mg/ml for leaves extract of *C. papaya*, *M. Piperita*, and fruit extract of *C. colocynthis*, respectively, in infected HepG2 cell line with 80-100 percent cell survival. However, ethanolic extracts 100 μ g/ml of thai medicinal plants *Cladogynosorientalis*, *Piper retrofractum* and *Rhizophoraapiculata* reported against DENV-2 inhibition by 52.9, 84.93 and 41.5% respectively using vero cell culture (Klawikkan et al., 2010). The author observe more viral inhibition at less concentration as compare to medicinal extracts used in

current study. It could be possible that extracts of thai medicinal plants (*C. orientalis* and *P. retrofractum*) have more active photochemical ingredients as compare to extracts used in current study.

According to Joseph et al. (2015), *C. papaya* leaf extracts in methanol/chloroform had cytotoxic concentrations (CC50) of 0.615 and 1 mg/ml in DENV-2 in LLC-MK2 cells. However, *C. papaya* leaf extract was less toxic to the HepG2 cell line with CC50 1.2 mg/ml compared to *M. Piperita* with CC50 0.6 mg/ml, while *C. colocynthis* fruit extract had the highest cytotoxicity with CC50 0.4 mg/ml compared to other extracts. In the present study, only methanolic *C. papaya* leaf extract was found to have a 50% cell survival rate in the HepG2 cell line, which was higher (1.2mg/ml) than the previous research (0.615mg/ml) in LLC-MK2 cell culture. The lower cytotoxicity in *C. papaya* leaf extracts in the previous study may be due to the different host culture cells.

Yucharoen et al. (2012) tested a methanolic extract of *M. piperita* against the HSV-2 virus in Vero cells and found that it inhibited the virus by less than 50%. In the current study, the methanolic extract of *M. piperita* only inhibited DENV-2 by 24.14 % percent,

which is 50% less activity than the previous study. The disparity in inhibitory effect may be because both experiments used different viruses and cell cultures. Mode of inhibitory action of *C. colocynthis* fruit methanolic extract may be by direct interaction and deactivation of dengue viral particals. This is similar to mode of action of the bactericidal effect of Flavonoids, alkaloidsof *C. colocynthis* against *Staphylococcus aureus*. In previous study 5 mg/mL *C. colocynthis* fruits ethanolic extract have a significant inhibitory effect with novobiocin against hospital isolated strains of *Staphylococcus aureus* (Najafi et al., 2010). Antiviral activity of *C. colocynthis* had not been reported yet in Pakistan.

Current study explore the potential of *C. colocynthis* to inhibit DENV-2 replication and can be used in drug therapy against dengue viruses. Further investigation to isolate the active ingredients or bioactive compounds from tumma fruit extract to inhibit dengue virus replication.

CONCLUSION

In conclusion, according to the results of a recent study, *C. colocynthis* can be used to prevent DENV-2 replication and may be used in dengue virus drug therapy. In comparison to the leaf

extracts of *C. papaya* and *M. piperita*, the methanolic fruit extract of *C. colocynthis* has the highest inhibitory activity against DENV-2 in the HepG2 cell line. It is possible to separate active ingredients or bioactive compounds from *C. colocynthis* fruit extract to prevent dengue virus replication for further research.

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Conflict of interest

Author's declare there is no conflict of interest.

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