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RESEARCH

Effect of methanolic-extracted green tea on hepatitis A virus and its usage in clearing the virus from drinking Water

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ABSTRACT

Background: Hepatitis A virus (HAV) causes tens of millions of human infection annually worldwide. It usually spreads by the fecal-oral route and is transmitted from person-to-person through contaminated food or water. A number of travelers arriving from Egypt were found infected with HAV upon return to their home countries, for this Egypt Travel made warned tourists traveling to Egypt to avoid eating under cooked or raw food (especially meat and eggs) or raw vegetables and drinking tap water.

Aim: To study potential antiviral effect of methanolic extract prepared from green tea on HAV and explore supplementing drinking water in endemic areas for the virus with such an extract to control infection.

Methods: Green tea powder was used for methanolic extraction. Cytotoxicity assay was carried out according to Aquino (1989) to identify non-cyotoxic concentrations of the prepared extract. Plaque reduction assay was performed to investigate the anti-HAV activity of the green tea extract according to Tebas (1995), evaluate its effect, to test effect of applying green tea extract against contaminated drinking water with HAV and study stability of inhibitory effect of extract on HAV in water.

Results: Green tea extract has a strong anti-HAV effect with direct effect on the viral particles. Supplementing tap water with the extract with continuous shaking every 15 minutes for 1 hour caused more reduction in the percentage of HAV plaque counts compared to allowing the supplemented water to rest for without shaking and the same effect was shown when water was freezed and tested after one and two weeks indicating stability and irreversibility of the inhibitory effect of extract on HAV in water.

Conclusion: Methanolic extract of green tea has potential effect on HAV. Based on the obtained results we strongly recommend supplementing water with small concentration of green tea extract that does not cause observed change in its color and taste for 1 hour with persistent shaking before usage in endemic areas for HAV infections.

Key words: Hepatitis A virus, Green tea, plaque reduction assay, drinking water treatment.

BACKGROUND

According to the World Health Organization (WHO), it is estimated that 1.4 million hepatitis A infections occur each year (WHO, 2000). . It usually spread by the fecal-oral route; transmitted from person to person through contaminated food or water (Wasley *et al*., 2006). HAV was found in drinking water (Ali *et al.*, 1997), waste water also has the virus (Schlindwein *et al*., 2009) not only that but also groundwater was reported as source of virus (Abbaszadegan *et al*., 2003, and (Borchardt et al., 2003).

HAV infection causes fever, malaise, weakness, anorexia, nausea, vomiting, arthralgias and myalgias (Koff, 1998), Wasley *et al.*, 2006). Fulminant hepatitis is a severe complication of

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hepatitis A virus infection (HAV). Its mechanism is unknown but spontaneous recovery is frequent. There are no data on the level of viral replication according to the clinical form of HAV (Rezende *et al*., 2003). A high fatality rate among chronic hepatitis B- or C-infected patients with HAV super-infection was observed (Lee, 2003).

Since November 2012, several European countries, including Denmark, Germany, the Netherlands, Norway, Sweden and United Kingdom have reported an increase in cases of hepatitis A virus (HAV) infections in travelers returning from Egypt. As in Apr 2013, Eightycases were reported in travelers who visited different areas of Egypt. Almost no cases were vaccinated prior to travel (MacDonald *et al.,* 2013).In 2004, a major outbreak of hepatitis A among tourists returning from Egypt involved 351 case-patients from 9 European countries who were infected with a single strain (genotype 1b) (Frank *et al*., 2007).

This situation leads to having Egypt Travel recommendations to tourists who are traveling to Sharm El Sheikh, Luxor, Alexandria, Aswan, Taba or Marsa Matrouh of Egypt to avoid eating less cooked or raw food (especially meat and eggs), drinking tap water and eating raw vegetables because Hepatitis A is common and it is wise to be vaccinated.

Not only in Egypt but also in USA also Community, restaurant, and school outbreaks due to contaminated water or food have been described (Fischer *et al.,* 2008).

The disease outbreaks due to contaminated water or food were reported. De Serres and his group showed that HAV RNA could be detected in well water six months after the initial contamination (De Serres *et al*., 1999).

Human wild-type HAV rarely grows in cell culture and requires several weeks to months in culture before it can be detected (Cohen *et al*., 1989). HAV has been adapted to a variety of primate (Binn *et al*., 1984), Daemer *et al*., 1981) and nonprimate (Dotzauer *et al.,* 1994), Feigelstock *et al.,* 2005) cell lines.

Mutant strains like HM175/18f that was adapted to grow on cell cultures leads to cellular degeneration and visible plaques when propagated in cell culture (Christian *et al*., 2000).HAV MBB strain was also adapted to grow on PLC/PRF/5 cell line (Reiner *et al*., 1992). Replication of the virus in diploid cells of human embryo fibroblasts, continuous primate lines (RAMT, FRhK-4) and human urinary bladder tumor (T-24) lines was also reported (Farashian *et al*., 1990)Adapted HAV strains enables scientists to test antiviral activity of many compounds like some pyrrolo[2,3-d]pyrimidines (Rashad *et al*., 2006), some sugar arylglycinoylhydrazones and their oxadiazoline derivatives (Abdel-Aal *et al*., 2006), synthesized triazolo[4,3-b]pyridazines (Shamroukh and Ali, 2008) and 5-(1,2,3-triazol-1-ylmethyl)uridine derivatives (Abdel-Rahman and Wada, 2009) and from plant extracts that were examined for their anti HAV MBB activity are *Dianthus caryophyllus L.* and *Lupinustermes*L. seed extracts (Barakat *et al.,* 2010).

In our study we examined the inhibitory effect of green tea methanolic extract on cell culture-grown adapted strain of HAV, and its antiviral effect on inoculated HAV in drinking water samples in a way can be used to treat tap waters in areas known to contain contaminated drinking water.

MATERIALS AND METHODS

Green tea extraction and preparation for bioassay:

A weight of 10 gram green tea powder from Egyptian market (Isis, Egypt) was macerated overnight in 60 ml methanol. Extract was filtered, methanol was evaporated till dryness and dry extract was stored at -20^{o}C until use (El-Menshawi, 2003).10 mg were resuspended in 1 ml of 10 % Dimethylsulfoxide (DMSO) in deionized, decontaminated by 1% antibiotic-antimycoticmixture.Sterile extract was aliquoted and stored at –20^{o}C till being used.

Virus:

A cell culture adapted HAV strain that was originally isolated from sewage sample in Egypt was provided by Dr. Ali Fahmy (The Holding Company for Biological Products & Vaccines of Egypt;VACSERA).This isolate was confirmed to be HAV by various molecular and serological means. Virus was amplified to final titer of 10^6 PFU/ml.

Cells:

Human hepatoma (HepG2) cells were brought from VACSERA and grown in DMEM (Lonza, USA) supplemented with 10 % fetal bovine serum (Gibco, USA), 1% antibiotic-antimycotic mixture (Lonza, USA).

Cytotoxicity assay:

This test was carried out to determine the safe doses of the green tea methanolic extract that can be applied over cells without harming them (Aquino *et al*., 1989). HepG2 cells were seeded in 96 well plate and incubated overnight, the extract was added to cells by the following concentrations : 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µg/100µl , this was followed by microscopic observation after 24 hours incubation to monitor any morphological changes to cells and select the safe doses of the extract.

Plaque reduction assay:

Plaque infectivity count assay is the most widely accepted method for determining the % inhibition of virus as a result of being subjected to a given material (Tebas *et al*, 1995). Amentadine was used as positive control as it is one of the antiviral substances known to interfere with HAV replication (Hollinger and Emerson, 2001). A 12 well plate was seeded with the HepG2 cells (10^5cell/mL) and incubated for 1 days at 37°C. Virus was diluted to final concentration of 10^6 PFU/mL and mixed with the safe concentrations of green tea extract (10-100µg/ml) and incubated for 1 h at 37°C. Growth medium was removed from the multi-well plate and virus extract mixture was inoculated over cells (100 µl/ well). After 1 h attachment time for virus adsorption, 2ml of cell specific 2× medium 2% agarose was overlaid the cell sheet. The plates were left to solidify and incubated at 37°C until development of the viral plaques. Formalin was added for two hours then plates were stained with crystal violet staining solution. Control virus and cells were treated identically without plant extract. Viral plaques were counted and the percentage of virus reduction was calculated.

Direct effect of extract on HAV particle:

For a compound to have antiviral effect with plaque reduction assay one cannot say if this result was due to direct effect of extract on the viralparticle or the extract affected viral adsorbtion or stopped any of its replication steps (Mohamed *et al.*, 2010) an so need further step to know the exact way the extract affected the virus.

To test direct effect of the extract on HAV, the virus was incubated with the compound and then ten fold dilution is made in a way to remove all extract but still there is convenient number of viral particles to continue through the assay so by this we became sure that the % of viral inhibition was due to direct effect on the viral particle and not due to effect on adsorbtion or any other replicating steps of the virus.

This was done in a 6 well plate was seeded with HepG2 cell (10^5 cell/ml) and incubated for 1 days at 37°C. A volume of 200 ml serum free DMEM containing 10^9 PFU/ml of HAV was added to different concentration of extract (10-50 µg/ml), after 1 h incubation, the mixture was subjected to 10 fold dilution 3 times in a way that leaves nearly no extract, 100 µL of each dilution was added to the HepG2 cell monolayer. After 1 h attachment time, assay was completed as mentioned above inn plaque reduction assay section (Schuhmacher *et al*., 2003)

Treatment of drinking water samples with green tea extract and testing stability of green tea effect on HAV:

To test inhibitory effect of green tea on HAV contaminating drinking water, drinking water was inoculated with known viral count (10^5 PFU/ml), treated with green tea at concentrations 10, 20 and 30 µg/ml and incubated at room temperature for 1 hour, samples were divided into two groups, the first was subjected to shaking every 15 minutes and the second was left stationary. A 12 well plate was seeded with the HepG2 cells (10^5cell/ml) and incubated for 1 days at 37°C.100µl from each water sample was inoculated over HepG2 cells and after 1 hour attachment time for viral adsorption, steps were completed as mentioned under plaque reduction assay section. Second part of experiment was to test stability and irreversibility of green tea action on HAV in water samples. Samples were stored at -20^{o}C and tested once after one week and once after two weeks.

RESULTS

Cytotoxicity test:

Results shown in table 1 showed that on applying green tea at different concentrations starting from 10 to 100 µg /100µl to epG2 cells and incubating it overnight, all concentrations used were found to be safe, although from 40 to 100 µgcaused some morphological changes than the control cells (untreated with extract) but these changes did not end with cell death so all tested concentrations can be used safely inn plaque reduction assay.

Table (1): Cytotoxicity effects of tested Green Tea-methanolic extract.

Concentration	10-40 µg/100µl	40-100 µg/100µl
Safety	Safe	Safe with morphological cell changes

Plaque reduction assay:

This test was carried out to test changes in viral count as a result of being subjected to different concentration of methanolic extract of green tea (from 10 to 100 µg/ml) and comparing its effect with Amentadine which is a positive control to viral inhibition at the same concentrations, Results in figure 1 showed that green tea gave about 80 % viral inhibition at concentration 10 µg/ml and 100 % inhibition starting from 20 µg/ml on the other hand Amentadine showed gradual increase in viral inhibition with increasing its concentration but it did not reach the same percentage of viral inhibition shown by the green tea extract.

Direct effect of extract on viral particle:

On applying the different safe concentrations of green tea extract (from 10 to 50 µg/ml) with the virus, then the one hour incubation was followed by ten fold dilution in a way that all the extract was removed but still a convenient viral count is found and then applied over cells. Results in figure 2 showed that the high percentage reduction exerted by green tea extract on the HAV is as a result of direct effect of extract on the virus and not due to any effect on viral adsprbtion or any step in its replicating cycle .

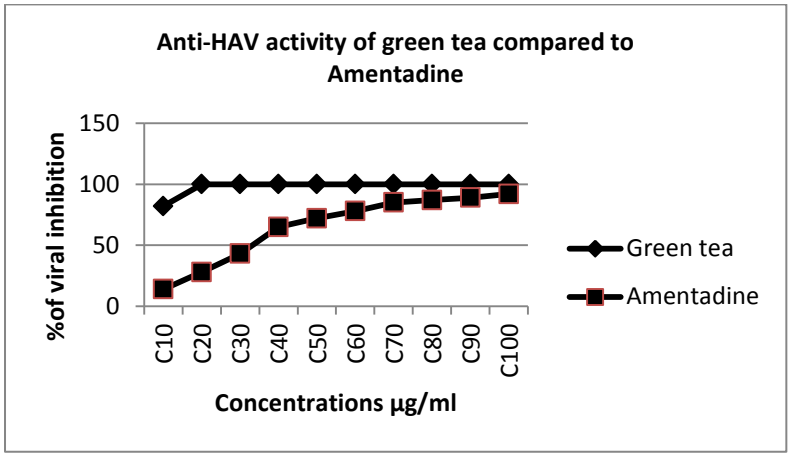

Figure (1): On applying Green Tea with concentrations from 10 -100 µg/ml on HepG2 cells to monitor the change in viral count and comparing it with Amentadine as positive control to viral inhibition, results showed that extract has high inhibitory effect on HAV that was even higher than that effect given by treating virus with the positive control.

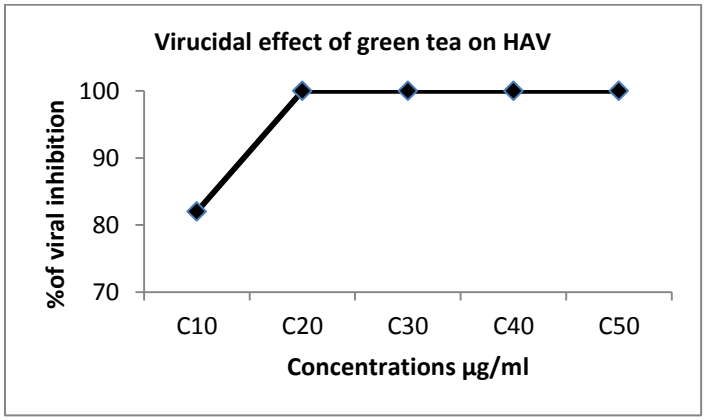

Figure (2): on applying green tea at concentrations 10 -50µg/ml on HAV, incubated then subjected to serial dilutions in a way that nearly no extract but convenient viral count were left, after applying that to cells results showed high percentage reduction at all the concentrations used indicating the direct effect of green tea extract on the viral particle causing its inhibition and loss of infectivity.

Treatment of water samples with green tea extract and testing stability of green tea effect on HAV:

This experiment was made to monitor the inhibitory effect of green tea on HHAV contaminating drinking water. Drinking water was inoculated with known count of HAV (10^5 PFU/ml) and divided into two groups one was subjected to shakinng every 15 minutes during 1 hour of incubation and the other was left stationary, results in Figure 3 showed that green tea was more effective when added to water subjected to shaking and gave 90 % inhibition at concentration 10 µg/ml and 100 % reduction at both 20 and 30 µg/ml which was not the same when water was left stationary as it gives 60, 72 and 80 % inhibition at 10, 20 and 30 µg/ml respectively. Second part of experiment was to test stability of inhibitory effect of green tea on HHAV in drinking water and if this effect is irreversible, results in Figure 4 showed that the inhibitory effect of

green tea on HAV was still active after one and two weeks of freezing of water indicating that the inhibitory effect was irreversible and not affected by freezing.

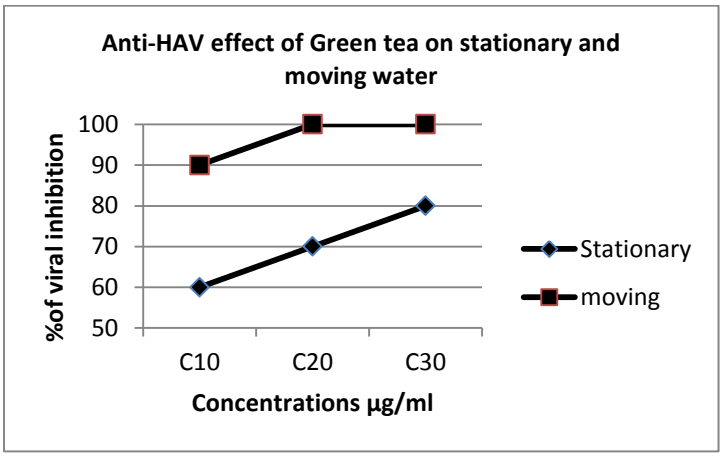

Figure (3): Green tea has higher inhibitory effect when applied in drinking water contaminated with HAV and subjected to shaking every 15 minutes for one hour which was higher than its effect on water left stationary during same incubation time.

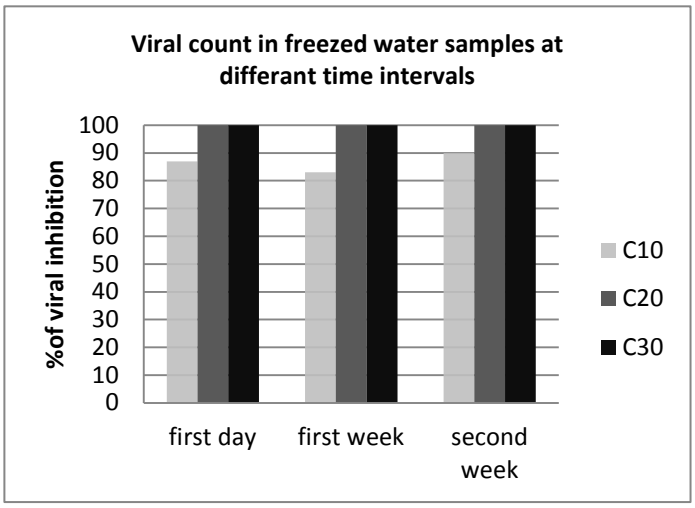

Figure (4): Inhibitory effect of green tea on HAV inoculated in drinking water was stable and irreversible through two weeks and its effect is not inhibited by freezing.

DISCUSSION

In this work we were trying to look for an extract that can be used to clear drinking water that might be contaminated with HAV and it was important for this extract to be safe and available in markets to be easily used by people in houses or hotels in areas famous of presence of contaminated water with the virus.

Grean tea is considered a healthful beverage due to the biological activity of its polyphenols namely catechism. Several epidemiological studies have proved that green tea also has some general health benefitting properties like antihypertensive, reduction of cardiovascular risk, antibacterial, antiviral, and antifungal activity (Venkateswara *et al.*, 2011). Green tea was reported to have antiviral effect against HSV-2 (Cheng *et al.*, 2002),

influenza A virus (mixed with other compounds) (Jariwalla *et al.*, 2007) and HCV (Ciesek *et al.*, 2011).

Usage of methanol for plant extraction was proven to be the best solvent in extracting phytochemical compounds like phenols, flavenoids and antioxidents (Dhawan and Gupta; 2017)

Cytotoxicity assay was carried out to determine the cell culture safe doses of the green tea methanolic extract (Aquino *et al.*, 1989). The test was made in 96 well plate seede with HepG2 cells, extract was inoculated to cells by 10, 20, 30, 40, 50, 60, 70, 80, 90, 100µg/100µl this was followed by microscopic observation after 24 hours incubation. Results (Table 1) showed that all concentration under test caused no cytotoxic effect on cells and so can be used on bioassays with great safety

Plaque infectivity count assay is the most widely accepted method for determining the % inhibition of virus as a result of being subjected to a given material (Tebas *et al.*, 1995). Amentadine was used as positive control as it is one of the antiviral substances known to interfere with HAV replication (Hollinger and Emerson, 2001). that green tea gave about 80 % viral inhibition at concentration 10 µg/ml and 100 % inhibition starting from 20 µg/ml on the other hand Amentadine showed gradual increase in viral inhibition with increasing its concentration but it did not reach the same percentage of viral inhibition shown by the green tea extract (Figure 1).. That confirm the high inhibitory effect of the extract on the virus

For an extract to have antiviral effect from plaque reduction assay experiment, this might be due to whether extract has direct effect on viral particle causing it to lose its infectivity, or affecting viral adsorbtion to host cell receptors or finally affecting any of replicating steps of the virus so stop its infectivity (Mohamed *et al.*, 2010).. Herein as we concentrated on inhibitory effect of green tea on HAV present in drinking water which has nothing to do with the cells that was why we concentrated in our experiments only on the direct effect of extract on the viral particle and not on adsorbtion or replication and results showed that green tea extract has direct inhibitory effect on HAV viral particle (Figure 2).

Treating water samples, inoculated with HAV at high viral count which can impossible be found in drinking water, with different concentration of extract showed that green tea remove 90 % of virus at concentration 10 µg/ml and on increasing concentration to 20 µg/ml all viral particles were inhibited.

This appeared on water subjected to shacking every 15 minutes during only 1 hour and this high effect was not shown in water samples left without shaking (Figure 3).

Water samples were freezed and tested three successive times (one hour after treatment, one and two weeks after treatment) where no change in results was observed indicating that binding of extract to the virus is irreversable and not affected by freezing the water(Figure 4).

We highly recommend water treatment with small dose of green tea extract, that will cause unobserved change in water color and taste, 1 hour with shaking before usage in areas famous with high % of HAV infection.

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