SOME MEDICINAL PLANT EXTRACTS EXHIBIT POTENCY AGAINST VIRAL HEPATITIS C

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ABSTRACT
Background: Hepatitis C virus (HCV) infection is gaining increasing attention as a global health crisis. HCV may lead to a substantial health and, consequently, economic burden over the next 10-20 years. Aim of the work: This research seeks to demonstrate antiviral activity of naturally derived extracts on HCV. Aqueous extracts of Milk thistle (MSE) as well as lyophilized juice of ginger were tested in-vitro at different concentrations (100, 200, 300, and 400μg/ml) using the HepG2 cell line infected with HCV. Inhibition of viral replication was detected by the amplification of viral RNA using the reverse transcriptase (RT)-PCR technique. Both the MSE and juice were considered active upon inhibiting the viral replication inside the HCV-infected HepG2 cells, as evidenced by the disappearance of the (+) and/or (-) strands of viral RNA-amplified products detected by RT-PCR (compared with the positive control). Results: The effective anti-viral dose was 100 μg/ml for Ginger and 300 μg/ml for MSE. Conclusion: This study provides results justifying preclinical evaluation of the tested extracts as antiviral therapy for HCV infections.

Keywords: Reverse transcriptase-PCR, Antiviral drug, Hepatitis C, Medicinal plants

1. INTRODUCTION
Hepatitis C virus (HCV), first identified in 1989 [1], is the major aetiological agent of non-A non-B hepatitis. The infection occurs principally through blood or blood-derived products and affects globally more than 270 million people [2]. Egypt reports the highest prevalence of HCV worldwide, ranging from 6% to more than 40% among different regions and demographic groups [3]. HCV frequently leads to chronic hepatitis and cirrhosis, in addition to being associated with the development of hepatocellular carcinoma [4]. In spite the highly vigorous and extensive research in this field, a protective vaccine and effective treatment are not yet available.

The mainstay of anti-HCV therapy, interferon (IFN-α) along with ribvirin leads, at best, to viral clearance for about 40-50% of patients infected with HCV [5]. Therefore, exploration for new anti-HCV principles is urgently needed. However, the absence of an efficient cell culture system to replicate HCV has prevented this trial for a long time. Recently, a model for in-vitro anti-HCV screening was established [6-10].

This circumstance prompted us to engage in search for unprecedented HCV invasion inhibitors from medicinal plants. Many herbal medicines possess antioxidant properties, which may play an important role in therapeutics [11-16]. Two of the most beneficial medicinal plants are Ginger and Milk thistle. Ginger (Zingiber officinale) has long been used in traditional medicine as a cure for some diseases including inflammatory diseases [17]. Ginger contains active phenolic compounds such as gingerol, paradol and shogoal which possess antioxidant [18] anti-cancer [19] anti-inflammatory [20&21] anti-angiogenesis [22] and anti-atherosclerotic properties[23]. Milk thistle has been reported to have protective effects on the liver and to greatly improve its function. It is typically used to treat liver cirrhosis, chronic hepatitis (liver...
inflammation), toxin-induced liver damage and gallbladder disorders [24&25].

The current work seeks to justify preclinical evaluation of the extracts of these natural products as antiviral therapy for HCV infections.

2. MATERIALS AND METHODS

2.1 supplies

Bulk ginger rhizome (from Zingiber officinale) was obtained from Metro supermarket, washed through with tap water, peeled and cross-sectional cut into 2±mm thickness in order to squeeze them to get the juice. Milk thistle (Silybum marianum) seeds (were obtained from a local market at Alexandria, Egypt. The seeds of Milk thistle were washed with water and air dried away from direct sunlight. Seeds were crushed in a coffee grinder for a total of 4 min at 15 s on &off intervals (actual grinding time: 2 min) to avoid heating the sample. The crushed seeds were wrapped and stored at -18°C until extraction [26].

2.2 HPLC analysis

The HPLC grade solvents, including methanol and phosphoric acid were obtained from Merek (Darmstadt-Germany) and nanopure water was used. The phenolic compounds standards were obtained from Sigma –Aldrich (St. Louis, Mo) and used as received. The analysis was done using a Beckman-C18 column (100X4.6 mm, 5um particle size), equipped with an auto sampler, quaternary pump and UV/Visible multi-wavelength detector. The components of the ginger and MSE samples were separated as described by National Science Foundation (NSF). The eluent A and eluent B for the three extracts was as follows:

For ginger, Eluent A: Water
Eluent B: acetonitrile

For MSE, Eluent A: 200 mL of methanol were mixed with 800 mL of water. 5 mL of phosphoric acid (85%, ACS Reagent grade) were mixed into 995 mL of the methanol: Water (20:80).

Eluent B: Mix 800 mL of methanol with 200 mL of water. Mix 5 mL of phosphoric acid into 995 mL of the methanol: Water (80:20).

2.3 Determination of phenol content

The classic technique employed in phenol analysis is the 4-aminoantipyrine colorimetric procedure [27]. The absorbance of the samples was read against blank at 500 nm using spectrophotometer (PerkinElmer Lambda EZ 201, USA). The concentration of the sample was calculated from the standard curve prepared previously.

2.4 Determination of antioxidant activity

The antioxidant activities of the herbs water extracts were determined using the ferric thiocyanate (FTC) method [28] with slight modification. Sample (20 mg) was dissolved in 4 mL of 95% (w/v) ethanol, then was mixed with 4.1 mL of linoleic acid [2.51% (v/v) in 99.5% (w/v) ethanol], 8 mL of 0.05 M phosphate buffer pH 7.0 and 3.9 mL distilled water and kept in screw-cap containers at 40°C in the dark. To 0.1 mL of this solution was then added 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured, and it was measured again every 24 h until the time when the absorbance of the control reached the maximum value.
The percent inhibition of linoleic acid peroxidation was calculated as:

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\text{% inhibition} = 100 - \left[ \frac{\text{absorbance increase of the sample}}{\text{absorbance increase of the control}} \right] \times 100
\]

All tests were run in duplicate, and analyses of all samples were run in triplicate and averaged.

2.5 Cell Culture

In the last few years, a number of cell culture systems have been developed that support reliable and efficient progression of this virus. Among several human hepatocyte cell lines analyzed, the hepatocellular carcinoma HepG2 cell line was found to be the most susceptible to HCV infection [29].

HepG2 cells were washed twice with RPM1640 media supplemented with 200µM L-glutamine and 25µM HEPES buffer; N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (all chemicals and media, Cambrex). The cells were suspended at 2X10^5 cells/mL in RPM1 culture media (RPM1 supplemented media, 10% fetal bovine serum (FBS); Gibco-BRL). The cells were left to adhere on the polystyrene 6-well plates for 4 h in an incubator (37°C, 5% CO₂, 95% humidity). The cells were washed twice from debris and dead cells by using RPM1 supplemented media.

2.6 Proliferative activities

Peripheral blood mononuclear cells (PBMC) were isolated from a healthy individual by Ficoll-Hypaque (Sigma, St.Louis,MO,USA) gradient centrifugation. The purified cells was cultured at 1.0X10^6 cell/mL in RPM1 complete medium (Camprex, Verviers, Belgium) supplemented with 25mM N-[2-hydroxyethyl] piperazine-N’-[2-ethanesulphonic acid] (HEPES) (Sigma), 4mM L-glutamine (Camprex), and 10% FBS (Gibco-BRL), PBMC were stimulated with 5,10,25,50,100,150,200, 250 and 300 µg/mL of each extract. All samples were assayed in triplicates. A positive control culture was included, where PBMC was stimulated with 2 µg/mL phytohemaglutinin-L (PHA, Sigma).

Proliferation was determined after incubation for 3 days at 37°C, 5% CO₂ and 95% humidity, by addition of 20 mL of BrdU labeling reagent (Roche, Penzberg, Germany) in the last 2 h of the culture. The labeled cultures were harvested and Brdu uptake was determined using Cell proliferation ELISA, Brdu (colorimetric) kit (Roche) following the manufactures instructions. Data were presented as stimulation index (SI), where proliferation is considered positive if SI is ≥ 2.

2.7 Qualitative in-vitro Anti-HCV Screening

PBMC and HepG2 cell culture were prepared as discussed, then infected with 2% HCV-infected serum in RPMI culture medium containing 8% FBS. Aqueous extract of MSE as well as lyophilized juice of ginger was added at concentrations of 5,10,25,50,100,150,200, 250 and 300 µg/mL. Positive and negative control cultures were included. After 96 h of incubation at 37°C, 5% CO₂ and 95% humidity, another dose of the tested extract was added. The cells were incubated for another 96 h at 37°C, 5% CO₂ and 95% humidity, followed by total RNA extraction. The positive strand and its replicating form (negative strand) were detected by RT-PCR using HCV specific primers to the 5’-untranslated region of the virus.

2.8 RNA extraction from PBMCs and HepG2 cells

Monitoring of the HCV viremia pre-and post-antiviral therapy through the detection
of viral (+) and /or (-) RNA strands by the use of qualitative RT-PCR has become the most frequently used, reliable and sensitive technique. Recently, it has been reported that the detection of the (-) strand HCV-RNA using the RT-PCR is a very important tool for understanding the life cycle of the HCV. It provides a reliable marker for the diagnosis of HCV and monitors the viral response to antiviral therapy [6].

Based on these facts, the adopted method in the present study contributes to the simultaneous detection of the (+) and /or(-) HCV-RNA strands isolated from peripheral blood mononuclear cells (PBMC) of infected patients as well as, HCV RNA isolated from HepG2 cells. RNA was isolated from PBMCs and HepG2 cells as described by Lohr et al. 1995 [30]. Briefly, cells were precipitated and washed in the same buffer to remove adherent viral particles before lysis in 4 mol/L guanidinium isothiocyanate containing 25 mmol/L sodium citrate, 0.5% sarcosyl and 0.1 mol/L |β|-mercaptoethanol and 100 μL sodium acetate. The lysed cells were centrifuged at 12000 rpm for 10 min at 4°C. The aqueous layer was collected and mixed with equal volume of isopropanol. After incubation at 20°C overnight, RNA was precipitated by centrifugation at 12000 rpm for 30 min at 4°C and the precipitate RNA was washed twice with 70% ethanol.

2.9 PCR of genomic and anti-genomic RNA strands of HCV

Reverse transcription-nested PCR was carried out according to Lohr et al., 1995 [30], with few modifications. Retrotranscription was performed in 25 μL reaction mixture containing 20 U of AMV reverse transcriptase (Clontech, USA) with either 400 ng of total HepG2 or PBMCs cells RNA, 40 U of RNAsin (Clontech, USA), a final concentration of 0.2 mmol/L from each dNTP (Promega, Madison, WI, USA) and 50 pmol of the reverse primer 1CH (for plus strand) or 50 pmol of the forward primer 2CH (for minus strand). The reaction was incubated at 42°C for 60 min and denatured at 98 for 10 min. Amplification of the highly conserved 5'-UTR sequences was done using two rounds of PCR with two pairs of nested primers. First round amplification was done in 50 μL reaction mixture, containing 50 pmol from each of 2CH forward primer and P2 reverse primer, 0.2 mmol/L from each dNTP, 10 μL from RT reaction mixture as template and 2 U of Taq DNA polymerase (Promega, USA) in a 1× buffer supplied with the enzyme. The thermal cycling protocol was as follows: 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 30 cycles. The second round amplification was done similar to the first round, except for use of the nested reverse primer D2 and forward primer F2 at 50 pmol each. A fragment of 174 bp was identified in positive samples. Primer sequences were as follows: 1CH: 5'ggtgcacggtetactacaggt-3', 2CH: 5'-aactactgtcttcacgcagaa-3', P2: 5'-tgctcatggtgcacggtcta-3', D2: 5'-actcggctagcagtctcgcg-3' and F2: 5'-gtgcagcctccaggaccc-3'. To control false detection of negative-strand HCV RNA and known variations in PCR efficiency, specific control assays and rigorous standardization of the reaction were employed: (1) cDNA synthesis without RNA templates to exclude product contamination; (2) cDNA synthesis without RTase to exclude Taq polymerase RTase activity; (3) cDNA synthesis and PCR step done with only the reverse or forward primer to confirm no contamination from mixed primers. These controls were consistently negative. In addition, cDNA
Fig. 1.
Resulting chromatogram and gradient elution schedule for HPLC-UV analysis of (A): Ginger, (B): Milk thistle Extract

Fig. 2.
Inhibition of linoleic peroxidation by ascorbic acid as a standard and ginger (A), and MSE (B), as measured by the FTC method. Absorbance values represent means of triplicates of different samples analyzed.
Fig. 3.
Dose response curve of tested extract effect on lymphocyte proliferation of healthy individuals. The data expressed as the Optical density of the BrdU incorporation assay as mentioned in materials and methods. All reading above dashed line are positive

![Graph showing dose response curve](image)

Fig. 4.
RT-PCR amplification products on gel electrophoresis: A, represents the PCR product of HCV RNA (+) and (−) strands isolated from HepG2 cells at different concentrations of ginger (1) and MSE (2). B, represents the PCR product of HCV RNA (+) and (−) strands isolated from peripheral blood mononuclear cells (PBMC) of infected patients at different concentrations of ginger (1) and MSE (2).

![Gel electrophoresis images](image)

3. RESULTS
3.1 HPLC
We adopted the HPLC method for analyzing the isolated compounds as well as screening the extracts. Fig. 1 showed high sensitivity and reproducibility, which agrees with the standard chromatograms produced by NSF. The resulting chromatogram A which illustrated in Fig.1 shows that the three major compounds in ginger were 6-gingerdiol, 6-gingerol and 10-gingerol. In the chromatogram C there were two major compounds taxifolin and silychristin. Taxifolin derivative is also present in a significant amount, as well, low amount of...
both silybinin A and B were also recorded. All compounds were identified by their retention times against standard samples. Other peaks in both chromatograms were not identified due to the lack of standards, they are most probably, phenolic compounds in a sense they contributed significantly to the total phenolics in the extract because total phenolics measured were much higher than the sum of the individual phenolic concentration identified and quantified by HPLC.

3.2 The phenolic content
Both extracts recorded high phenolic content. It was 40.1% for ginger and 77.3% for MSE.

3.3 The antioxidant activity
Figure 2 shows the inhibition of linoleic peroxidation by ginger and MSE respectively in comparison with ascorbic acid as a standard.

3.4 The immuonomodulatory effect
The overall immuonomodulatory profiles of, ginger and MSE enhanced lymphocyte proliferation at concentrations up to 200 µg/ml, Figure 3. These results demonstrate that not only do the extracts show no cytotoxicity effect but they also augment lymphocyte proliferation (immuno-stimulatory effect) in a dose dependant manner.

3.5 Anti-viral effect
Inhibition of viral replication was detected by amplification of the viral RNA segments using RT-PCR technique. The test extract is considered to be active when it is capable of inhibiting the viral replication inside The HCV-infected cells, as evidenced by the disappearance of the (+) and /or (-) strands RNA amplified products detected by the RT-PCR (compare to positive control).

Out of the tested extracts, lyophilized juice of ginger proved to inhibit HCV replication completely at concentration 100µg/ml, while MSE inhibited HCV replication at concentration 300µg/ml as shown in fig. 4.

4. DISCUSSION
Usually, screening of antiviral compounds has been snagged due to the lack of representative smaller animal model. However, in the case of HCV infection in humans, vector-based assay techniques have been very helpful in screening a variety of antiviral agents [31], but measuring the viral RNA synthesis in the absence and presence of the agent in an efficient cell culture system remains important. Real time PCR has proven to be accurate for determination of antiviral drug susceptibility of herpes viruses [32&33]. The real time PCR assay was used in the current study to determine whether the HCV–RNA replication is inhibited in infected cells (PBMC and HepG2) by the plant extract or not.

Our data showed that lyophilized juice of ginger caused complete inhibition of intracellular viral replication at 100µg/ml. This confirms the previous investigation done by Sookkongwaree et al, 2005 [34], where they reported the inhibition of HCV NS3 protease by ginger extract. They expressed the full length of HCV NS3 protease and then they purified and started the assay at enzymatic level. The second extract tested in the current study was the MSE, showing an inhibitory effect at 300µg/ml. We have two records about the antiviral effect of MSE, the first is the clinical study done by Ferenci et al, 2008 [35], where they stated that silibinin ( one of the main constituents of MSE) is a potent antiviral agent in patients with chronic HCV not responding to pegylated interferon/ribavirin therapy. The second record is the in vitro study done by Bonifaz et al., 2009 [36]. They concluded that the silymarin (MSE) significantly downregulated HCV core
mRNA (by 20%–36%) and protein (by 30%–60%) in CNS3 cells. Our current study confirms those previous studies, and emphasizes the importance of screening of several herbs aiming identifying an anti-infectious agent offer a great potential in the field of pharmaceutical developments.

5. CONCLUSION
It can be fairly concluded that lyophilized juice of ginger and MSE have a great potential as sources for novel lead compounds with specific antiviral properties. Further bioassays guiding separation and isolation of the active compounds is therefore of interest. Finally, it is also of interest to determine whether the inhibitory effects are due to a synergistic effect of more than one effective compound or due to the sole effect of a highly potent compound. Such studies require more extensive kinetic studies using the pure compounds.

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7. REFERENCES


