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Original Article

Anti HCV activity and expression inhibition of HCC markers by protein extract from *Iberis gibraltarica*

Atividade anti-HCV e inibição da expressão de marcadores HCC por extrato de proteína de *Iberis gibraltarica*

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Abstract

Hepatitis C virus infection (HCV) is the foremost reason of progressive hepatic fibrosis and cirrhosis, with an elevated risk of hepatocellular carcinoma (HCC) development. Medicinal plants have been used for human health benefits for several years, but their therapeutic potential needs to be explored. The main objective of this study was to figure out the *in vitro* antiviral and anticancer characteristics of total crude protein of *Iberis gibraltarica* against HCV and HCC. Total crude protein of *Iberis gibraltarica was* isolated and quantified. The level of cytotoxicity was measured against the HepG2 cell line and it shows no significant cytotoxicity at the concentration of 504µg/ml. The anti-HCV effect was determined by absolute quantification via real time RT-PCR method and viral titer was reduced up to 66% in a dose dependent manner against the total protein of *Iberis gibraltarica*. The anticancer potential of *Iberis gibraltarica* was also examined through mRNA expression studies of AFP and GPC3 genes against the total protein of *Iberis gibraltarica*-treated HepG2 cells. The results show up to 90% of the down-regulation expression of AFP and GPC3. The obtained results indicate the therapeutic potential of total protein of *Iberis gibraltarica* against HCV and hepatocellular carcinoma *in vitro*.

Keywords: HCV, HCC, Iberis gibraltarica, HepG2 and Real time PCR.

Resumo

A infecção pelo vírus da hepatite C (HCV) é a principal causa de fibrose hepática progressiva e cirrose, com risco elevado de desenvolvimento de carcinoma hepatocelular (HCC). As plantas medicinais vêm sendo utilizadas para benefícios à saúde humana há vários anos, mas seu potencial terapêutico precisa ser explorado. O principal objetivo deste estudo foi descobrir as características antivirais e anticancerígenas in vitro da proteína bruta total de *Iberis gibraltarica* contra HCV e HCC. A proteína bruta total de *Iberis gibraltarica* foi isolada e quantificada. O nível de citotoxicidade foi medido contra a linha celular $HepG_2$ e não apresenta citotoxicidade significativa na concentração de 504µg/ml. O efeito anti-HCV foi determinado por quantificação absoluta através do método RT-PCR em tempo real e o título viral foi reduzido em até 66% de forma dose-dependente contra a proteína total de *Iberis gibraltarica*. O potencial anticancerígeno de *Iberis gibraltarica* também foi examinado através de estudos de expressão de mRNA dos genes AFP e GPC_3 contra a proteína total de células $HepG_2$ tratadas com *Iberis gibraltarica*. Os resultados mostram até 90% da expressão de regulação negativa de AFP e GPC_3. Os resultados obtidos indicam o potencial terapêutico da proteína total de *Iberis gibraltarica* contra HCV e carcinoma hepatocelular in vitro.

Palavras-chave: HCV, HCC, *Iberis gibraltarica*, $HepG_2$ e PCR em tempo real.

1. Introduction

One of the foremost bases of the chronic liver diseases is the hepatitis C virus (HCV), a hepatotrophic RNA virus. HCV is a small, enveloped, single-stranded, positive-sense RNA virus and a associate of the family *Flaviviridae* with a genome size of 9.6 kb. Among the different subtypes of HCV, genotype 3a is more prevalent in Pakistan (Alecu et al., 1998). About 170 million people around the world are believed to have been infected with HCV (Umer and Iqbal, 2016). HCV causes various diseases of the liver, which account for half a million deaths annually (Curry et al., 2015). There is currently no HCV vaccine available and combination therapy, such as pegylated interferon and ribavirin, has previously been used for HCV treatment (Manns et al., 2006), but not for all HCV genotypes (Palumbo, 2011). Direct-acting antivirals (DAAs) have been introduced for the management of chronic HCV. Boceprivir and telaprevir have significantly improved HCV treatment by inhibiting HCV NS3/4A proteases, although

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these drugs have shown serious side effects (Shaikh and Shih, 2012). More recently, sofosbuvir and ledipasvir have been approved for HCV treatment with improved results and have shown 96-99% sustained virologic response in 12 to 24 weeks (Scott, 2018). But the high cost of HCV care leaves a large number of HCV-infected people out of control, most of whom are in resource-poor countries such as South East Asia and Africa with very high burdens on HCV-positive people (WHO, 2017). Interferon and DAAs have many severe side effects (Sa-Ngiamsuntorn et al., 2016) however, viral resistance strains called Resistance Associated Strains emerge due to the heterogeneous pool of HCV, which explain their resistance to DAA treatment (Loggi et al., 2018).

The major complication of HCV virus infection is hepatocellular carcinoma (HCC) with significant rates of mortality and morbidity (Chen and Morgan, 2006). The prevalence rate of HCC is very high and accounts for 75% of cases of hepatic cancer (Jemal et al., 2011; Maillard, 2011). In addition, more than 80% of this cancer has also surfaced in developing economies where HCC prognosis is poor, with some other factors (Rehman et al., 2011) such as metabolic disorders such as metabolic syndrome, non-alcoholic fatty liver disease, obesity and diabetes increasing. (Yang and Roberts, 2010). Systematic treatment solutions for HCC included liver transplantation, chemotherapy, radiation and percutaneous medications, but these treatments remain largely inadequate. A variety of chemotherapy drugs against HCC have been reported (Peters et al., 2002). Besides, these drugs have also been associated with their side effects that are harmful to normal cells. Medicinal plants have been shown to reduce the threat of HCC and liver cirrhosis (Umer and Iqbal, 2016). Most of the research has been focused on developing more effective anti-viral and anticancer agents with selective toxicity (Senthilraja and Kathiresan, 2015, Vijayan et al., 2004). The number of plants with anticancer activity has been reported, but few of them include *Cannabis sativ*a (Galve-Roperh et al., 2000), *Bolbostemma paniculatum* (Cheng et al., 2006), *Apis mellifera* (Hamzaoglu et al., 2000), *Astralagus hedysarum* (Wang et al., 1989), *Hypericum perforatum (*Alecu et al., 1998) and *Camellia sinensis* (Esghaei et al., 2018) . *Iberis gibraltarica* relates to the genus *Iberis*, a member of the *Brassicaceae* family (Saeed et al., 2021). *Iberis amara* extracts have been reported to show anticancer activity (Weidner et al., 2016). The main objective of the current study was to examine the anticancer and antiviral potential of *Iberis gibraltarica* crude protein extract.

2. Materials and Methods

2.1. Total protein isolation

Iberis gibraltarica seeds were brought by the local seller (MOREGREEN, 2017) and cultivated in the CAMB field and subsequently verified by Dr. Zahoor Sajid (University of the Punjab). Three different buffers (Kim et al., 2011) such as Buffer 1; 100mM citrate buffer (pH3.0) containing 50mM NaCl, Buffer 2; 100mM acetate buffer (pH5.0) containing 50mM NaCl and Buffer 3 was comprised of 100mM phosphate buffer (pH7.0) containing 50mM NaCl were used to isolate the total crude protein. Seeds were ground to a fine powder with a pestle and mortar and mixed with each buffer in a proportion of 1:10 (w/v) for 2 hours at 4°C. Muslin cloth was used to remove the seed debris, and the supernatant was collected by centrifugation (Eppendorf 5415R, Germany) at 4°C for 30 minutes at 13000 rpm.

Bradford method was performed to find out the total protein concentration using Bradford Reagent (Bio-Rad, USA) (Bradford, 1976). Collected supernatant of individual pH was dialyzed separately overnight by dialyzing tube (Spectra/Por RC Biotech membrane, 6-8 kDa MWCO, USA) against 10mM of citrate buffer pH3.0, phosphate buffer of pH 7.0 and acetate buffer pH 5.0 at 4°C. Total protein isolated from distinct buffers was analyzed by SDS-PAGE (Smith, 1984).

2.2. Cell culture

The HepG2 cells were cultured using the Dulbecco's modified Eagle medium (DMEM) containing 100U/ml penicillin, 100 µg/ml streptomycin and 10% Fetal Bovine Serum (FBS) at 37°C in 5% CO2 incubator at 37°C in the cell culture lab, CAMB.

2.3. Cytotoxicity assay

The level of cytotoxicity of the protein extract was measured by the MTT (3-(4,5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide) assay kit (Millipore, USA). DMEM was used to culture 100µl (1×10^5) of HepG2 cells in 96 wells flat bottom plate (Corning, USA). Cells were grown at 37°C for 24 hours in a CO_2 incubator (BINDER, Germany). Different concentrations of the protein were supplemented to the 96-wells plate and then placed in a CO_2 incubator for 24 hours at 37 °C. For each dilution, three replicates were analyzed. The media was carefully removed from the wells and changed to 100µl of newly prepared media after 24 hours, as directed by the manufacturer, 10µL MTT solution (5mg/ml in PBS) was supplemented and incubated again in a CO_2 incubator at 37°C for 4 hours. Subsequently, wells were filled with 0.1ml of DMSO (Dimethyl sulfoxide) to dissolve the formazen crystals. The optical density of the MTT formazan product was taken by an ELISA (Enzyme-linked immunosorbent assay) reader (SpectraMax Plus 96, Molecular Devices, USA) with a reference wavelength of 620 nm and a test wavelength of 570 nm (Rehman et al., 2011).

2.4. Anti-HCV activity

HepG2 cells (3×10^5 cells/well) were grown in the 60mm cell culture dish. After attaining the 60-70% confluency, washed the cells with 1×PBS and infected with 500µl HCV (3a genotype) infected sera (1×10^5 IU/well) along with 500µl serum free media for 24 hours (El-Awady et al., 2006; Noreen et al., 2015). On the following day, wash the adherent cells with PBS twice and left the cells to grow for 48 hours.

In order to analyze the effects of total protein of *I.gibraltarica*, the infected HepG2 cells were left to grow in a 6-well plate. At the 60-70% confluency, treated the cells with different concentrations of total protein of

I.gibraltarica (30, 60 and 90µg/ml) along with the control (without protein) for 24 hours. The total RNA was then isolated from each well using the Qiagen RNA isolation kit as instructed by the manufacturer. Viral quantification was performed using the Qiagen HCV quantitative analysis kit. For viral RNA quantification, 10µl of the total RNA extracted from cell lysate was mixed with inhibitory concentration (IC) (described above) and quantified with Rotor-Gene Q 2plex HRM System (Qiagen, USA).

2.5. Relative quantification of AFP and GPC3 gene expression

HepG2 cells were seeded at a density of 1×10^5 in a 12-well plate and incubated at 37°C for 24 hours. Subsequently, the media was replaced and inoculated for another 24 hours with different concentrations (30, 60 and 90µg / ml) of crude protein extracts along with a protein-free buffer control in the CO2 incubator. Total RNA was extracted from protein-treated cells using the TRIzol Reagent (Invitrogen, USA) (Rio et al., 2010). Quantification of the extracted RNA was performed with the Nano drop (ND-1000, OptiPlex, USA) followed by synthesis of cDNA through the Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania) as instructed by the manufacturer.

2.6. Real time PCR analysis

The Alpha-fetoprotein (NM_001134) and Glypican 3 (NM_004484) mRNA expressions were evaluated in the protein-treated cells using real time PCR (PikoReal™ Thermo Fisher, Finland). β- Actin (NM_001101) was used as a reference gene (Li et al., 2010). Gene expression was analyzed by real-time PCR analysis with Maxima SYBR Green qPCR Master Mix (Thermo Scientific). For *AFP* RT-PCR amplification, one cycle of 95°C for 5 minutes was followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Similarly, for *GPC*3 RT-PCR amplification, one cycle of 95°C for 5 minutes was followed by 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. The relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. Table 1 (Supplementary material) lists the primers that were applied.

2.7. Statistical analysis

GraphPad Prism 7 software was used for statistical analyses. Cytotoxicity and anti-HCV experiments were carried out in triplicate, while *AFP* and *GPC*3 relative expression studies were performed in duplicate. The mean and standard deviations of the data were determined using descriptive statistics. One way ANOVA was used to explore the significant reduction in mRNA expression of the *AFP* and *GPC3* genes at $P\leq0.05$.

3. Results

3.1. SDS-PAGE analysis

In order to evaluate the maximum protein profile, the total protein was isolated by means of three distinct buffers (citrate buffer (pH3.0), acetate buffer (pH5.0) and phosphate buffer (pH7.0). Figure 1 (Supplementary material) shows the results of the SDS-PAGE for *Iberis gibraltarica* total protein. The total crude protein profile obtained by SDS-PAGE at three different pH levels is shown in Figure 1A. Figure 1B (Supplementary material) illustrates the silver staining results from three different pH levels. SDS-PAGE results from crude extracts revealed that maximum protein extraction was achieved at pH7.0, as indicated by the maximum number of protein bands and silver staining results. The pH7.0 buffer was therefore used to further isolate the protein from *I.gibraltarica*.

3.2. Cytotoxicity assay

The cytotoxic effect of *Iberis gibraltarica* total protein is shown in the Figure 2. The most considerable effect was achieved by up to 84% cell viability after 24 hours, with a maximum concentration of *I. gibraltarica* total protein of 504 µg/ml. A higher protein concentration can also be used for a more pronounced effect. Hence, no significant decrease in cell viability with respect to control was observed after 24 hours.

3.3. Anti -HCV Activity of I.gibraltarica

Total protein of *I.gibraltarica* was evaluated for antiviral activity against the HCV. The results obtained after real time RT-PCR shows that total protein of *I.gibraltarica* reduces the virus RNA for 20, 48 and 66% at the concentration of 30, 60 and 90µg/ml respectively, as shown in the Figure 3.

3.4. AFP and GPC3 gene expression

A major decrease in *GPC3* and *AFP* gene expression was observed in cells treated with *I.gibraltarica* complete protein. Delta delta method was used for qPCR analysis.

Table 1. List of primers used for the real time PCR study.

Primers	Sequences	Amplicon size
AFP Forward	5/-TGTCCCTCCTGCATTCTCTG-3/	189
AFP Reverse	5/-TGGCAGCATTTCTCCAACAG-3/	
GPC3 Forward	5/-TACTGCTCTTACTGCCAGGG-3/	186
GPC3 Reverse	5/-ACCAAGCAGTACGTTCTCCA-3/	
β-Actin Forward	5/-CCCTGGAGAAGAGCTACGAG-3/	180
β-Actin Reverse	5/-CGTACAGGTCTTTGCGGATG-3/	

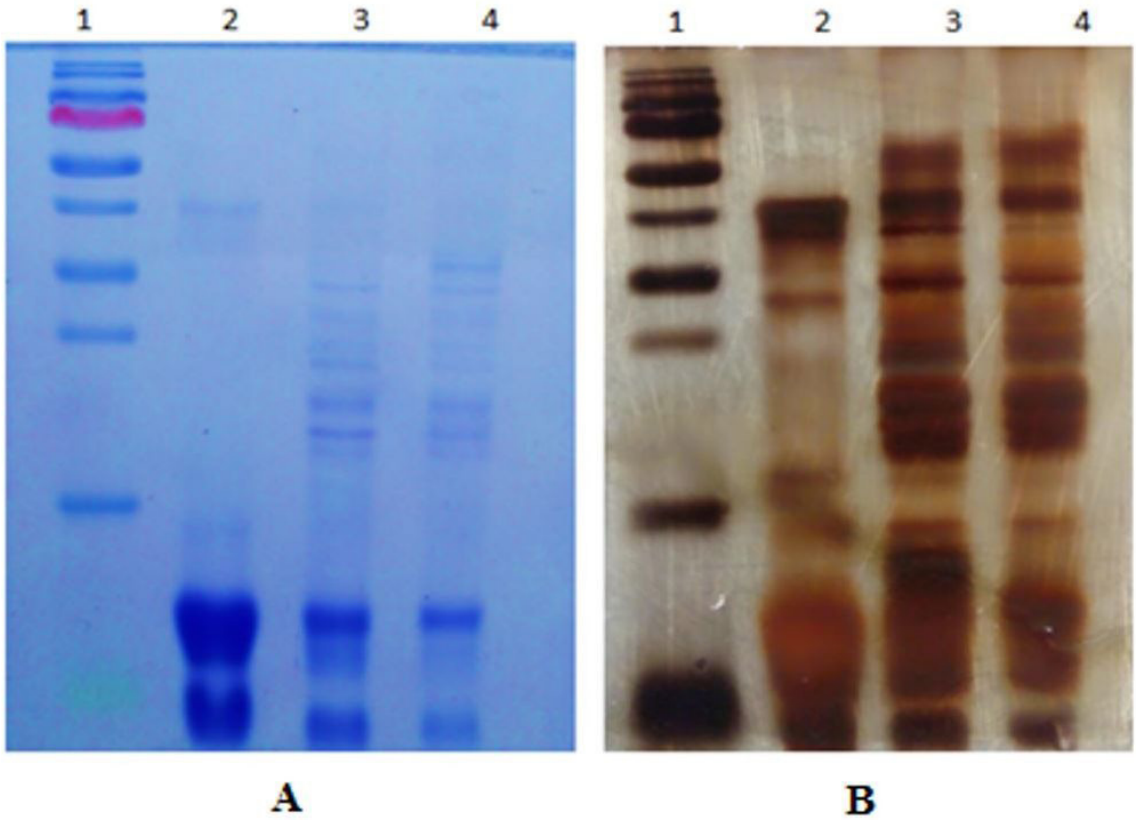

Figure 1. (**A**) indicates the SDS-PAGE analysis of *I.gibraltarica* total protein using three different buffers. Lane 1 represents the protein marker (Cat. No. 26616, Thermo, Lithuania). Lanes 2, 3 and 4 represent total protein isolated by citrate buffer (pH3.0), acetate buffer (pH5.0) and phosphate buffer (pH7.0) respectively; (**B**) represents the silver staining.

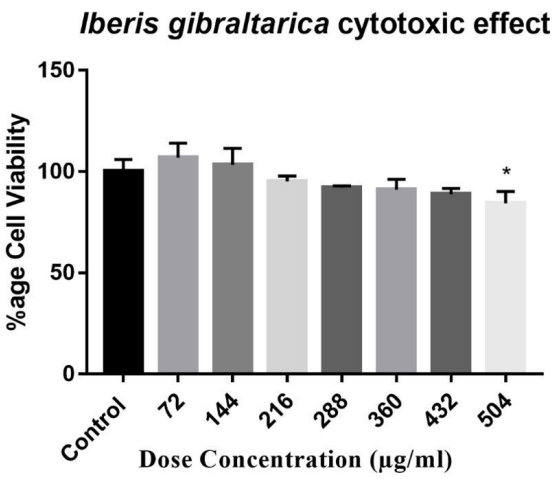

Figure 2. Cytotoxic effect of total protein of *I.gibraltarica* in HepG2 cells.

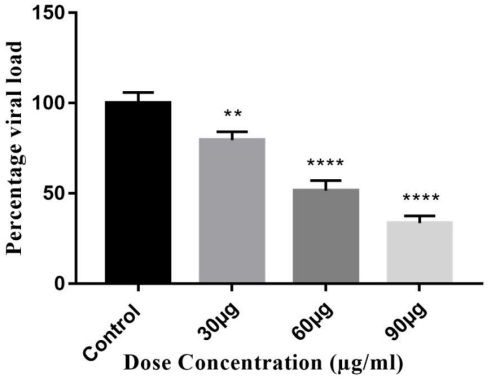

Figure 3. Antiviral effect of total protein of *I.gibraltarica* against HCV in HepG2 cells (liver cells). Real time PCR profile generated, and bars in the graph depict the standard deviation while, asterisks (**) indicates the $P<0.01$ and asterisks (****) indicate a significant decrease at $P<0.0001$ in HCV titer at 30, 60 and 90µg/ml doses of the total protein of *I.gibraltarica*.

Decreased *AFP* expression was up to 92%, while *GPC3* expression was observed in a dose-dependent manner as shown in Figure 4.

Melting curve analysis showing the melting temperature peaks (Tm) of AFP and GPC3 genes in Figure 1S and 2S, respectively.

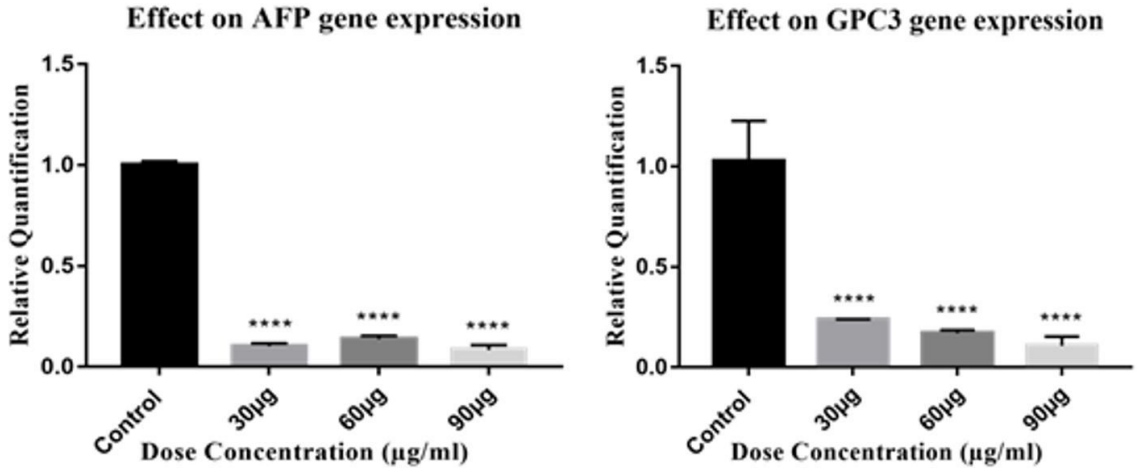

Figure 4. Effect of total protein of *I.gibraltarica* on *AFP* and *GPC3* gene expression. Real time PCR profile generated, and bars in the graph depict the standard deviation while, **** indicate a significant decrease at $P<0.0001$ in *AFP* and *GPC3* gene expressions at 30, 60 and 90µg/ml doses of the total protein of *I.gibraltarica*.

One way ANOVA was applied, and it shows that all the results obtained are statistically significant at $P<0.0001$.

4. Discussion

Plant-based substances such as small molecular compounds and proteins, have been proposed as effective medicines for cancer management in response to many of the adverse effects of conventional cancer treatments, such as chemotherapy and radiation therapy (Hernandez-Ledesma et al., 2013). A number of plants proteins are recognized for their anticancer and antiviral nature, a plant lectin purified from *Praecitrullus fistulosus* has been observed to inhibit tumor growth *in vivo (*Shivamadhu et al., 2017) and an antiviral protein (RIP type I) isolated from *Phytolacca americana* significantly inhibited the levels of TMV in *Nicotiana benthamiana* plant leaves. However, there are numerous plants that should be evaluated for their activities as Pokeweed, because Pokeweed contains some toxic components, and its therapeutic potential should be carefully evaluated (Zhu et al., 2016).

The maximum protein profile and concentration obtained at pH 7 buffer through SDS-PAGE and Bradford assay using Bradford Reagent (BioRad, USA) . Similar studies were carried out with bioactive protein extractions from different plant sources (Zekri et al., 2009) . Previously, using the hydroethanolic extract of *I.amara* has been examined against the colon cancer (Weidner et al., 2016).

Presently, no information is available on the bioactivity of *I.gibraltarica* seed protein extract. This study is the first antiviral and anticancer report on the potenial of *I.gibraltarica* seed proteins. The cytotoxic behavior of total protein isolated from *I.gibraltarica* was assessed using the HepG2 cells. The least cell viability percentage was measured at a concentration of 525µg/ml after 24 hours. The cytotoxicity was induced in a concentration dependent manner. Similar results have been observed in the protein extract of *Morinda pubescens*, which shows the maximum cell viability of the Vero cells at a concentration of 100µg/ml (Thomas et al., 2017). Another protein known as Galanthus nivalis agglutinin showed a minimal inhibitory effect on murine and human cancer cell lines when tested for cytotoxicity (Sandström et al., 2004).

Various plant species associated with different taxonomic families are found to generate endogenous, non-stress-induced inhibitor proteins called antiviral proteins (AVPs) (Choudhary et al., 2008). Certain plant proteins have demonstrated antiviral activity against a variety of plant viruses (Bilal et al., 2020; Lusvarghi and Bewley, 2016). Different plant protein such as lectins and ribosome inactivating proteins (RIPs) have also been shown the antiviral activities against the human infected viruses (Alecu et al., 1998; Al-Sohaimy et al., 2007). We evaluated the *in vitro* anti-HCV potential of the total protein of *I. gibraltarica*in hepatitis C virus infected HepG2 cells (Tang et al., 2008). The results of the Real Time RT-PCR show the total *I. gibraltarica* protein reduces the RNA virus level up to 66% at 90µg/ml. Similar results have been reported using the plant proteins against HCV *in vitro* studies (Al-Sohaimy et al., 2007; Ashfaq et al., 2011). Plant-based chemoprevention and treatment have evolved into useful and effective methods for the treatment and controlling cancer (Pongthanapisith et al., 2013). Plant peptides and proteins are among the most recent natural products with significant anti-carcinogenic activity (Guarneri and Conte, 2004).

The elevated levels of *AFP* has also been identified as the primary cause of HCC (Henderson et al., 2003) and the ability of its mRNA as a marker in HCC tumor cells has been used for reverse transcription PCR (RT-PCR) (Debruyne and Delanghe, 2008). AFP targeting with siRNA was considered to be a potential therapeutic approach to HCC (Tang et al., 2008). Various studies have shown that GPC3 protein facilitates the spread of HCC cells through the formation of a Wnt complex (Wingless-related integration site) (Ho et al., 2007). (Capurro et al., 2005). In HCC patients, GPC3 translational management offers an

effective clinical option (Debruyne and Delanghe, 2008). Furthermore, GPC3 silencing has also been documented to stimulate cell apoptosis (Sun et al., 2011). In this study, relative quantification of AFP and GPC3 genes was carried out in *I.gibraltarica* crude protein treated HepG2 cells. Real-time PCR findings represent a significant dose-dependent control of AFP and GPC3 expressions (Ruan et al., 2011). Similarly, significant decreases in AFP and GPC3 levels have also been reported in the treatment of *Gingko Biloba* leaf extract (Wang et al., 1989) . The *I.gibraltarica* crude protein cytotoxic values indicated the safe limit and worth for further testing (Abushouk et al., 2017). Therefore, purified individual protein/s from total protein of *I.gibraltarica* may be useful as antiviral and anticancer agents (Liu et al., 2012).

5. Conclusion

The total protein of *I. gibraltarica* was isolated and evaluated its antiviral and anticancer potential *in vitro*. Our results show that the total protein of *Iberis gibraltarica* exhibits significant antiviral activity and down-regulates HCC biomarkers. Our initial findings are very prominent and may open up a new event for further investigation of the individual protein(s) of *I. gibraltarica* as a natural potent therapeutic candidate for viral and cancer treatment.

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Supplementary Material

Supplementary material accompanies this paper.

Figure 1S. Melting curve analysis showing the melting temperature peaks (Tm) of AFP gene
Figure 2S. Melting curve analysis showing the melting temperature peaks (Tm) of GPC3 gene

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