Journal of Medical Microbiology and Infectious Diseases

JoMMID
ISSN: 2345-5349
eISSN: 2345-5330

The Effect of Silibinin on the Expression of TLR7, ISG15, and SOCS1 in Peripheral Blood Mononuclear Cells of Hepatitis C Infected Patients in Comparison with Interferon-α

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ARTICLE INFO

Original Article

Keywords: HCV, Silibinin, Interferon, ISG15, SOCS1

Received: 11 Oct. 2020
Received in revised form: 28 Feb. 2021
Accepted: 28 Feb. 2021
DOI: 10.52547/JoMMID.9.1.12

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ABSTRACT

Background: Silibinin (silibinin A) is the most active silymarin component, which acts both as a hepatoprotective [1] and an antiviral agent. The present study investigated the silibinin effect on IFN-related innate immune genes in PBMCs from HCV-infected patients. **Method:** 22 chronic HCV patients, including 10 IFN responders and 12 non-responders, were included. Their isolated PBMCs were treated for 6 hours in the presence of silibinin, IFN-α, or their combination. The transcription level of TLR7, ISG15, and SOCS1 genes was compared using real-time PCR. **Result:** Our result showed that IFN-α induced a significant up-regulation of TLR7 and ISG15 in PBMCs of both responder and non-responder groups. Nevertheless, the SOCS1 gene was not significantly changed in the non-responder group (P=0.32). The combination of IFNα- and silibinin showed a similar pattern to IFN-α alone. By itself, silibinin did not leave a significant change on the expression level of the studied genes . **Conclusion:** The results indicated that silibinin did not enhance or suppress the expression level of TLR7, ISG15, and SOCS1 genes. Therefore, it has been suggested that its anti-inflammatory role might be devoid of IFN pathways.

INTRODUCTION

Hepatitis C virus (HCV) infection affects ~1% of the world population [2, 3]. In about 55%-85% of HCV cases, persistent infection leads to cirrhosis and hepatocellular carcinoma [4, 5]. For a long time, the standard therapy for HCV infection was recombinant interferon-alpha (IFN-α) [6] though it is now mostly replaced by revolutionary and more effective direct-acting antivirals (DAAs) [7].

Among the suggested herbal medicines for HCV treatment, the flavonoids mixture (silymarin) from the milk thistle plant (*Silybum marianum*) is the most popular one and is considered safe and effective [8]. Silibinin (silibinin A) is the most active silymarin component, which acts both as a hepatoprotective [1] and an antiviral agent. Studies using Huh7.5.1 host cells have shown that silibinin displays a direct inhibitory role in the HCV replication cycle [9].

The hepatoprotective effects of silibinin partly depend on its suppressive role on activated stellate cells, which prevent fibrosis and cirrhosis development [10]. Also, silibinin suppresses the mRNA expression of proinflammatory cytokines [11]. Moreover, silibinin was able to partially reduce the levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and inflammatory cytokine in peripheral blood mononuclear cells (PBMCs) isolated from preeclampsia women [12]. However, the detailed mechanism for silibinin anti-inflammatory effect in PBMCs remains to be elucidated [13].

IFN-stimulated gene (ISG) 15, toll-like receptor 7 (TLR7), and suppressor of cytokine signaling1 (SOCS1) genes induced in response to IFN [14, 15] are among ISGs of the innate immunity [13]. The SOCS1 expression, a suppressor of the JAK-STAT pathway that increases in non-responsive patients, might be related to immune subversion [16]. TLR7 that detects single-stranded RNA could induce an innate antiviral activity in the host [17]. Also, ISG15, as a critical ISGs gene induced by IFN, exhibits an antiviral activity against viruses such as HCV and hepatitis B virus (HBV) [18, 19].

[DOI: 10.52547/JoMMID.9.1.12]

Further utilization of silibinin in viral-related inflammation requires a true understanding of its innate immunomodulatory role on PBMCs [20]. However, its synergistic or antagonistic effect on IFN related immunity on PBMCs from different HCV patients has not yet been investigated. Therefore, herein three IFN-α related genes in HCV patients, including SOCS1, TLR7, and ISG15, were investigated to determine if silibinin could modulate an IFN-induced innate immunity. To this end, we employed PBMCs groups treated with either IFN-α, silibinin, or a combination of IFN-α and silibinin.

MATERIAL AND METHODS

Patients. Overall, 22 chronic HCV-infected patients with genotype 1, including 12 non-responders and 10 responders, admitted to the Liver Clinic of Gasterohepatology Research Center, Shiraz University of Medical Sciences, were enrolled consecutively from 2013 to 2015. The informed consent under the university approval (Ethics committee approval No: 7721) was obtained, while an ethical guideline from Shiraz University of Medical Sciences was thoroughly considered. None of the participants received DAA drugs while they went through a course of peg-IFN-α plus ribavirin therapy. The identification of viral genomic RNA six months after the end of therapy was defined as non-response.

PBMCs culture and treatment. Blood samples were taken in heparin tubes, and fresh PBMCs were isolated by the Ficoll-Hypaque method (Inno-train Inc., Germany). The freshly isolated PBMCs (10^6 cells/well) were then split into 6-well plates and fed by a complete RPMI medium (Gibco Inc.) containing 10% FBS and penicillin-streptomycin. The PBMCs were treated with silibinin (silibinin group), recombinant IFN-α (IFN group), their combination (silibinin+IFN group), or nothing (negative control) and incubated in the air humidified CO_2 incubator until the peak of the expression change (6 hours).

Silibinin ($C_{25}H_{22}O_{10}$ MW= 482.44, Sigma-Aldrich Inc., USA) was dissolved in dimethyl sulfoxide (BioIdea Inc. Iran) and incomplete RPMI. It was added at a concentration of 50 µg/mL to one PBMCs group. Recombinant IFN-α 2b (3×10^6 IU/mL) (Pooyesh Daru Inc., Iran) was also added to the PBMCs group in a concentration of 500 IU/mL. IFN-α was used to show the extent of the immune response in PBMCs following stimulation in the presence or absence of silibinin.

In the third group, a combination of IFN-α and silibinin was added into relevant wells. A negative control group was also included, which was untreated. Before starting the experiment, the Trypan-Blue exclusion test [21] was employed to determine the cell viability/cytotoxicity after treatment with several doses of silibinin (50-1000µg/mL) and IFN-α (100-2000IU/mL) to find the optimal dose for further assessment.

Real-time PCR and gene expression analysis. Total RNA was extracted using an RNA extraction solution (Cinnaclone Inc. Tehran, Iran), according to the manufacture's instruction. The expression patterns of TLR7, SOCS1, and ISG15 were measured using real-time PCR assay by SYBR Green dye. The sequences of primers are shown in table 1. The reaction components used in this study have been described elsewhere [22]. The expression level of each target gene was determined in duplicate and normalized to the expression level of the human ACTB (actin, beta) reference gene.

Table 1. The list of primers employed in specific qPCRs

Gene	Primers
β-actin (ACTB)	Forward: 5′-GCCTTTGCCGATCCGC-3′ Reverse: 5′-GCCGTAGCCGTTGTCG-3′
TLR7	Forward: 5′-GAAAGTTGATGCTATTGGG-3′ Reverse: 5′-TTTGTCTCTTCAGTGTCC-3′
SOCS1	Forward: 5′- TTCGCCCTTAGCGTGAAGATGG-3′ Reverse: 5′- TAGTGCTCCAGCAGCTCGAAGA-3′
ISG15	Forward: 5′- TCATCTTTGCCAGTACAGGAGC-3′ Reverse: 5′- TTCTGGGTGATCTGCGCCTT-3′

Statistical Analysis. Data were analyzed using Graph Pad Prism version 5 (San Diego, California). The significant difference between means was determined by Mann–Whitney's U test or unpaired t-test, one-way ANOVA, and Kruskal-Wallis. P-values less than 0.05 were considered to be statistically significant.

RESULTS

Subjects. The characteristics of the patients are shown in Table 1.

Cytotoxicity results. In accordance with cytotoxicity results determined by the simple trypan blue exclusion test, concentrations of 50 µg/mL silibinin and 500 IU/mL IFN-α 2b were determined to be safer and more effective for further assessment. These doses showed less than 5% cell death in the PBMC population when quantified by the trypan blue exclusion test.

[DOI: 10.52547/JoMMID.9.1.12]

The effect of silibinin on PBMCs gene expression. The results of the expression pattern of SOCS1, TLR7, and ISG15 genes are shown in Fig. 1. Overall, these results showed a somewhat similar trend following IFN-α with or without silibinin treatment in both responder and non-responder groups. As a positive control, IFN-α induced a significant up-regulation of studied genes in PBMCs (P<0.01) among responders. However, the exception was SOCS1, which did not significantly change in the non-responder group (P= 0.32). Adding silibinin to culture media did not induce or suppress the innate inductive role of IFN-α, as shown in Fig. 1.

Table 2. The characteristics of the patients

	Responder	Non-responder
Male	8	9
Female	2	3
Mean age	42.9	50
Total	10	12

PBMCs treated with IFN-α showed a significant up-regulation for ISG15 in both groups, as expected. The increasing fold for ISG15 expression was higher than other genes (P<0.001). Hence, combining IFN-α + silibinin caused a significant elevation in the expression level of ISG15 (P<0.05). On the contrary, silibinin alone did not increase the expression of ISG15 in both groups.

In the case of TLR7, its transcription was up-regulated significantly following IFN-α treatment in both groups (P<0.001). Moreover, after treating PBMC with IFN-α + silibinin, the expression significantly increased in both groups (P< 0.01). Similarly, after treating PBMCs with silibinin, no significant change in the expression level of TLR7 was detectable, as measured for ISG15.

SOCS1 transcription analysis showed a different pattern from those of ISG15 and TLR7. After IFN-α treatment, the induction of SOCS1 expression was merely detected in the responder group (P<0.05). The PBMCs of the non-responder group did not respond to IFN-α treatment as SOCS1 expression showed similar levels before and after treatment. Adding silibinin to IFN-α showed no difference in comparison to single IFN-α treatment. Silibinin alone did not change the expression of SOCS1, as shown in Fig. 1 for other genes.

DISCUSSION

Even with discovering a new generation of DAAs in HCV therapy, the future treatment seems not to be completely free of additional components like silymarin [7, 23]. Silibinin can reduce inflammatory changes following DAAs therapy, restrict the fibrosis process, and slow down the HCC progression [24].

Our result showed that silibinin at the concentration tested is safe as it does not affect cell viability even when combined with IFN-α. Moreover, as a positive control and potent innate immunity stimulator, recombinant IFN-α treatment significantly induces all three gene transcription, except SOCS1 in the non-responder group. IFN-α could induce SOCS1 expression merely in PBMCs of the responder group. This might reflect upon the higher integrity of innate immune signaling of PBMCs among the responder group, affecting IFN-related responses [14]. For ISG15 and TLR7, expression augmentation following IFN-α alone or in combination with silibinin was detected in both groups. MacParland *et al*. (2015) reported that IFN-α showed a high induction level of TLR7 and ISG15, which was in line with our study [25].

On the contrary, silibinin, by itself, did not enhance the expression of the studied genes. Moreover, it has been reported that silibinin dose-dependently impairs the production of Th1 related cytokines such as secretion of TNF-α, IFN-γ, IL-4, and IL-2 from HCV-infected and uninfected subjects while increasing IL-10 in murine hepatocytes [26, 27]. In contrast, decreased levels of IL-10, IFN-γ, and TNF-α following silibinin treatment have also been reported [28]. Silibinin was also shown to significantly reduce transcription factors such as NF-κB and STAT1 [29]. On the other hand, no significant change in ISGs expression of basal lymphocyte was observed for silymarin treatment. However, as the silymarin dose increased, more robust interferon-induced ISG15 expression was observed [30]. It has been reported that slight alterations of ISGs expression were observed following silymarin therapy in a dose-dependent pattern [30].

When a combination of IFN-α and silibinin was applied, no significant amelioration of the IFN-mediated innate induction was detected, which suggested the non-modulatory role of silibinin. In a dozen experiments, silibinin has exhibited an anti-inflammatory effect through different pathways [26]. It has been firmly suggested that this property is related to blocking the NF-κB signaling pathway, resulting in the down-regulation of pro-inflammatory cytokines [26] and through dendritic cell modulation [20]. In persistent HCV infection, inflammation is mainly induced through the NF-κB pathway, resulting in fibrosis and antiviral resistance [31]. As Lovelace *et al*. showed, silymarin suppresses HCV-induced oxidative stress, pro-inflammatory mRNAs, T cell activation, and signaling pathways, including NF-κB following prolonged (i.e., 24 h) exposure [11]. Consequently, despite different mechanisms known for the anti-inflammatory role of

[DOI: 10.52547/JoMMID.9.1.12]

silibinin, the possible impact on IFN-related innate immunity remains well-established.

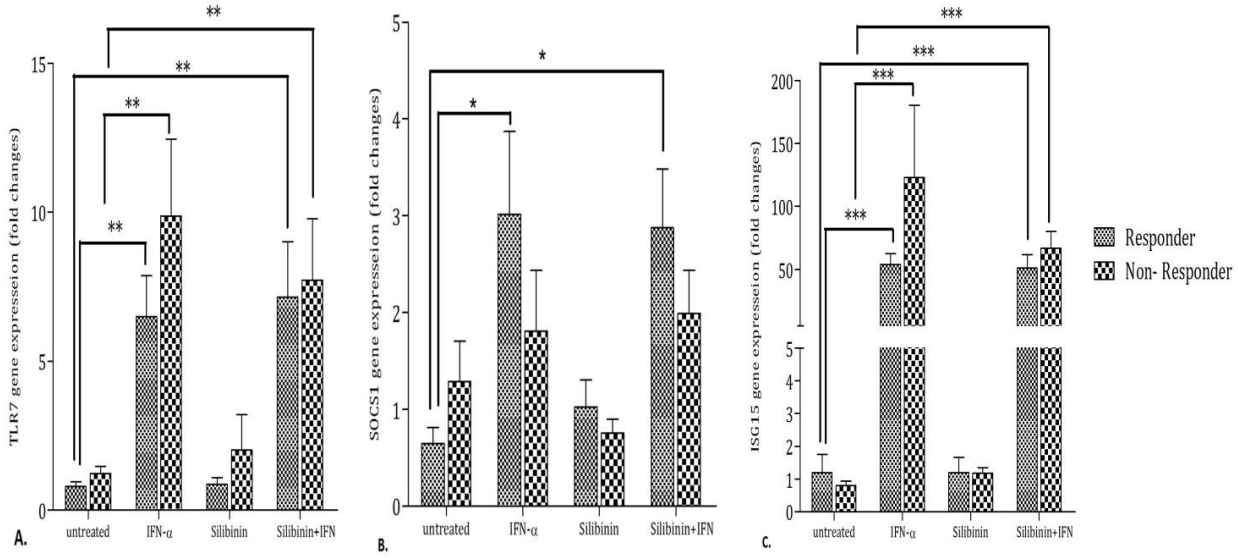

Fig. 1. The transcription level of the three genes A) TLR7, B) SOCS1, and C) ISG15 in responder and non-responder groups in four culture conditions. Error bars represent the standard error of the mean. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Silibinin might not have a stimulatory or suppressive effect on IFN-related genes, including TLR7, ISG15, and SOCS1, and exhibited no contra-indicatory effect with IFN-α. Therefore, its anti-inflammatory role seems to be devoid of IFN pathways. However, this suggestion should be confirmed through more IFN-related gene expression analysis, including normal cell and multiple-dose regimens of silibinin.

ACKNOWLEDGMENT

This work was supported by Shiraz University of Medical Sciences, Grant No. 93-01-13-7721. The authors thank Dr. Kurosh Kalantar and Dr. Zahra Amirghofran for their assistance and advice. The authors wish to thank Mr. H. Argasi at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his invaluable assistance in editing this manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests associated with this manuscript.

REFERENCES

1. Loguercio C, Festi D. Silybin and the liver: from basic research to clinical practice. World J Gastroenterol. 2011; 17 (18): 2288-301.

2. Pradat P, Virlogeux V, Trepo E. Epidemiology and Elimination of HCV-Related Liver Disease. Viruses. 2018; 10 (10): 545

3. Sarvari J, Mansouri M, Hashempoor T, Hosseini SY, Moattari A, Pirbonyeh N, Dowran R, Moayedi J, Musavi Z, Fattahi M-R. Association of genotype and haplotype of IL-28B Gene with Hepatitis C infection outcome in Iran: Spontaneous clearance versus chronic infection. Hepat Mon. 2017; 17 (5): e45745.

4. Barathan M, Mohamed R, Yong YK, Kannan M, Vadivelu J, Saeidi A, et al. Viral Persistence and Chronicity in Hepatitis C Virus Infection: Role of T-Cell Apoptosis, Senescence and Exhaustion. Cells. 2018; 7 (10): 165.

5. Sarvari J, Dowran R, Hosseini SY, Fattahi MR, Erfani N. Association of PD-1 gene with outcome of hepatitis C virus infection. EXCLI J. 2018; 24 (17): 935-44.

6. Sarvari J, Moattari A, Pirbonyeh N, Moini M, Hosseini SY. The Impact of IFN-gamma Gene Polymorphisms on Spontaneous Clearance of HCV Infection in Fars Province, Southern of Iran. J Clin Lab Anal. 2016; 30 (4): 301-7.

7. Patel M, Rab S, Kalapila AG, Kyle A, Okosun IS, Miller L. Highly Successful Hepatitis C Virus (HCV) Treatment Outcomes in Human Immunodeficiency Virus/HCV-Coinfected Patients at a Large, Urban, Ryan White Clinic. Open Forum Infect Dis. 2017; 4 (2): ofx062.

8. Ferenci P, Scherzer TM, Kerschner H, Rutter K, Beinhardt S, Hofer H, Schoniger-Hekele M, Holzmann H, Steindl-Munda P. Silibinin is a potent antiviral agent in patients with chronic hepatitis C not responding to pegylated interferon/ribavirin therapy. Gastroenterology. 2008; 135 (5): 1561-7.

9. Polyak SJ, Morishima C, Shuhart MC, Wang CC, Liu Y, Lee DY. Inhibition of T-cell inflammatory cytokines, hepatocyte NF-kappaB signaling, and HCV infection by standardized Silymarin. Gastroenterology. 2007; 132 (5): 1925-36.

10. Hellerbrand C, Schattenberg JM, Peterburs P, Lechner A, Brignoli R. The potential of silymarin for the treatment of hepatic disorders. Clin Phytoscience. 2016; 2 (1): 7.

[DOI: 10.52547/JoMMID.9.1.12]

11. Lovelace ES, Wagoner J, MacDonald J, Bammler T, Bruckner J, Brownell J, et al. Silymarin Suppresses Cellular Inflammation By Inducing Reparative Stress Signaling. J Nat Prod. 2015; 78 (8): 1990-2000.

12. Giorgi VS, Peracoli MT, Peracoli JC, Witkin SS, Bannwart-Castro CF. Silibinin modulates the NF-kappab pathway and pro-inflammatory cytokine production by mononuclear cells from preeclamptic women. J Reprod Immunol. 2012; 95 (1-2): 67-72.

13. Liu B, Chen S, Guan Y, Chen L. Type III Interferon Induces Distinct SOCS1 Expression Pattern that Contributes to Delayed but Prolonged Activation of Jak/STAT Signaling Pathway: Implications for Treatment Non-Response in HCV Patients. PLoS One. 2015; 10 (7): e0133800.

14. Piganis RA, De Weerd NA, Gould JA, Schindler CW, Mansell A, Nicholson SE, et al. Suppressor of cytokine signaling (SOCS) 1 inhibits type I interferon (IFN) signaling via the interferon alpha receptor (IFNAR1)-associated tyrosine kinase Tyk2. J Biol Chem. 2011; 286 (39): 33811-8.

15. Khanlari Z, Sabahi F, Hosseini SY, Ghaderi M. HCV NS3 Blocking Effect on IFN Induced ISGs Like Viperin and IL28 With and Without NS4A. Hepat Mon. 2014; 14 (6): e17822.

16. Younossi ZM, Baranova A, Afendy A, Collantes R, Stepanova M, Manyam G, et al. Early gene expression profiles of patients with chronic hepatitis C treated with pegylated interferon-alfa and ribavirin. Hepatology. 2009; 49 (3): 763-74.

17. Dehghan-Manshadi M, Hadinedoushan H, Amirbaigy MK, Zare F, Eslami G, Mirghanizade-Bafghi SA, et al. Relative Expression of Toll-Like Receptors 2 and 7 mRNA in Peripheral Blood of Patients With Hepatitis C. Hepat Mon. 2015; 15 (11): e30427.

18. Chen L, Li S, McGilvray I. The ISG15/USP18 ubiquitin-like pathway (ISGylation system) in hepatitis C virus infection and resistance to interferon therapy. Int J Biochem Cell Biol. 2011; 43 (10): 1427-31.

19. Hashemi SMA, Sarvari J, Fattahi MR, Dowran R, Ramezani A, SY H. Comparison of ISG15, IL28B and USP18 mRNA levels in peripheral blood mononuclear cells of chronic hepatitis B virus infected patients and healthy individuals. Gastroenterol Hepatol Bed Bench. 2019; 12 (1): 38-45.

20. Castellaneta A, Massaro A, Rendina M, D'Errico F, Carparelli S, Rizzi SF, et al. Immunomodulating effects of the anti-viral agent Silibinin in liver transplant patients with HCV recurrence. Transplant Res. 2016; 5: 1.

21. Khodadad M, Hosseini SY, Shenavar F, Erfani N, Bina S, Ahmadian S, et al. Construction of expressing vectors including melanoma differentiation-associated gene-7 (mda-7) fused with the RGD sequences for better tumor targeting. Iran J Basic Med Sci. 2015; 18 (8): 780-7.

22. Dowran R, Sarvari J, Moattari A, Fattahi MR, Ramezani A, Hosseini SY. Analysis of TLR7, SOCS1 and ISG15 immune genes expression in the peripheral blood of responder and non-responder patients with chronic Hepatitis C. Gastroenterol Hepatol Bed Bench. 2017; 10 (4): 272-7.

23. Manns MP, Wedemeyer H, Cornberg M. Treating viral hepatitis C: efficacy, side effects, and complications. Gut. 2006; 55 (9): 1350-9.

24. Braun DL, Rauch A, Aouri M, Durisch N, Eberhard N, Anagnostopoulos A, et al. A Lead-In with Silibinin Prior to Triple-Therapy Translates into Favorable Treatment Outcomes in Difficult-To-Treat HIV/Hepatitis C Coinfected Patients. PLoS One. 2015; 10 (7): e0133028.

25. MacParland SA, Corkum CP, Burgess C, Karwowska S, Kroll W, Michalak TI. Differential expression of interferon alpha inducible genes in peripheral blood mononuclear cells from patients chronically infected with hepatitis C virus and healthy donors. Int Immunopharmacol. 2015; 25 (2): 545-52.

26. Esmaeil N, Anaraki SB, Gharagozloo M, Moayedi B. Silymarin impacts on immune system as an immunomodulator: One key for many locks. Int Immunopharmacol. 2017; 50: 194-201.

27. Schumann J, Prockl J, Kiemer AK, Vollmar AM, Bang R, Tiegs G. Silibinin protects mice from T cell-dependent liver injury. J Hepatol. 2003; 39 (3): 333-40.

28. Das SK, Mukherjee S. Biochemical and immunological basis of silymarin effect, a milk thistle (Silybum marianum) against ethanol-induced oxidative damage. Toxicol Mech Methods. 2012; 22 (5): 409-13.

29. Hou YC, Liou KT, Chern CM, Wang YH, Liao JF, Chang S, et al. Preventive effect of silymarin in cerebral ischemia-reperfusion-induced brain injury in rats possibly through impairing NF-kappaB and STAT-1 activation. Phytomedicine. 2010; 17 (12): 963-73.

30. Adeyemo O, Doi H, Rajender Reddy K, Kaplan DE. Impact of oral silymarin on virus- and non-virus-specific T-cell responses in chronic hepatitis C infection. J Viral Hepat. 2013; 20 (7): 453-62.

31. Almasi E, Gharagozloo M, Eskandari N, Almasi A, Sabzghabaee AM. Inhibition of Apoptosis and Proliferation in T Cells by Immunosuppressive Silymarine. Iran J Allergy Asthma Immunol. 2017; 16 (2): 107-19.

Cite this article:

Dowran R, Hosseini SY, Fattahi MR, Sarvari j. The Effect of Silibinin on the Expression of TLR7, ISG15, and SOCS1 in Peripheral Blood Mononuclear Cells of Hepatitis C Infected Patients in Comparison with Interferon-α. J Med Microbiol Infect Dis, 2021; 9 (1): 12-16. DOI: 10.52547/JoMMID.9.1.12

[DOI: 10.52547/JoMMID.9.1.12]