

***In vitro* evaluation of antiviral activity of *Sisybrium irio* (Khaksi) against SARS-COV-2**

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Abstract: SARS-CoV-2 pandemic, drawn attention to the need of virus culture. *In vitro* SARS-COV-2 culture was performed to carry out therapeutic, environmental and virus genome studies. Isolation of virus from nasopharyngeal swab was performed by inoculating virus positive samples in available cell lines. SARS-CoV-2 topography was observed by using Scanning Electron Microscopy (SEM). Virus specificity was defined by serological confirmation through neutralization assay with COVID 19 convalescent sera. The SARS-COV -2 virus replicated successfully in Vero cell lines (both in E6 and CCL-81). The TCID₅₀ and PFUs of isolated virus were defined as 10⁷ TCID₅₀/mL and 1.4 X 10⁶ pfu/mL respectively. The virus particles with the SARS-CoV morphology was ≤150nm size. Virus inhibition in presence of convalescent sera of COVID-19 patients was observed. *Sisybrium irio* (Khaksi) was found cytotoxic on Vero E6 cell line and its antiviral activity could not be established against SARS-COV-2 virus *in vitro*. Successful isolated and archived native SARS-COV-2 may be utilized further for therapeutic, environmental and virus genome sequencing studies.

Keywords: Coronavirus, SARS-COV-2, COVID 19, virus isolation, virus neutralization, antiviral activity, *Sisybrium irio* (Khaksi).

INTRODUCTION

In December 2019, Wuhan City, Hubei Province, China get a new introduction to the world as a city reportedly with the circulation of a new virus causing severe acute respiratory syndrome. The virus was found similar as SARS virus in China outbreak of 2002-03. The virus was given the name as novel corona virus 2019 (nCoV2019) later on virus was classified as SARS-COV-2 and disease was termed COVID-19. On 30th January World Health Organization (WHO) declared COVID a global emergency and pandemic. Till February 2020 virus reached many countries of the world including Pakistan. The first two cases of Pakistan were confirmed on 26th February 2020 with the travel history from Iran.

Two other coronavirus major outbreak were happened in Past SARS in 2002-03 in China and Middle East Respiratory Syndrome MERS in Saudi Arabia 2012. Earlier the other coronavirus seropositivity (MERS) was reported in Pakistan but not the circulating virus (Zohaib *et al.*, 2018; Saqib *et al.*, 2017; Zheng *et al.*, 2019).

SARS-CoV-2 suspected samples diagnosis was started in CESAT diagnostic lab on 31st March 2020 that later on became the source of virus isolation. The study aimed to isolate the novel pandemic causing pathogen from nasopharyngeal swab in laboratory setting to perform

research and therapeutic studies. Virus isolation attempts were made from the patient's samples. SARS coronavirus isolation was initially reported on non-primate kidney cell lines as VeroE6 and FRhK (Ksiazek, *et al.*, 2003; Peiris *et al.*, 2003). Later on virus growth on Vero CCL81 and hSLAM (Araujo *et al.*, 2020; Harcourt *et al.*, 2020; Sarkale *et al.*, 2020). Vero CCL81, Vero E6, Huh 07 and BHK 21 were used for this purpose. Cytopathic Effect (CPE) was noticed as cytoplasmic inclusion of the cells followed by rounding off the cells and clearance followed by detachment of monolayer. Real-time PCR was used as detection tool for virus growth. The selected, isolated virus was used to make high titer archiving, further passages, electron microscopic SEM examination, determination of TCID and plaque assay, neutralization of virus with SARS-CoV-2 convalescent sera and therapeutic studies.

Sisybrium irio having common name of London rocket or Khaksi is easily available in market. It is well known for its broad spectrum therapeutic application in unani medicine system (Hailu *et al.*, 2019). It belongs to family Brassicaceae or Cruciferae. Moreover, silver nanoparticles of the *Sisybrium irio* leaves shown to be highly effective against gram negative nosocomial infection causing bacteria (Mickymaray 2019; Farag *et al.*, 2021). Earlier crude extracts of *Sisybrium irio* was found to have antipyretic, analgesic, antibacterial effects with both gram positive and negative bacteria and antifungal (Vohora *et al.*, 1980; Javaid *et al.*, 2017). While

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computing the effect of *Sisybrium irio*, as an antipyretic agent, its use is suggested as co therapy to treat COVID (Ansari *et al.*, 2020; Iqbal *et al.*, 2021). Water, ethanol and Dichloromethane extract of Khaksi were utilized to test against SARS-COV-2 in the laboratory to see if there is some direct antiviral effect of this herb on SARS-COV-2.

MATERIALS AND METHODS

Khaksi was procured from a herbalist Store “Hakim & Sons”, Karachi. Identification of Khaksi was performed by Hakim Abdul Bari, Hamdard University, Pakistan. according to “The Unani Pharmacopodia of India”. Sample Collection and Preparation Nasopharyngeal swab specimens found COVID positive with CT ≤ 20 were selected to perform SARS-COV-2 culture. The samples were provided in viral transport medium. Samples were centrifuged at 1200-1500 RPM for 10 minutes. Collected supernatant were diluted 1:2 with serum free DMEM, followed by filtration through 0.2 μ syringe filter.

Cell Lines available cell lines Vero CCL8, Vero E6, BHK-21 and Huh07 and cell lines were harvested for virus isolation. All cell lines monolayers maintenance were performed in growth medium DMEM (Invitrogen), supplemented with 10% fetal Bovine Serum- FBS (Invitrogen) keeping at 37°C, humidity 88rh and 5% CO₂. These incubation conditions were kept constant in all later experiments.

Virus Isolation was performed by inducing infection in all above mentioned cell monolayers with the 1 ml diluted, filtered sample extracts without trypsin and incubated for an hour. Cell culture Flasks were slanted to and fro after 10 minutes interval. Samples were removed and cells were washed with 3ml serum free DMEM thrice followed by 5 ml addition of reduced serum free (1% Serum) DMEM for 72 hours. Virus isolation experiments were executed in BSL 3 facility with good laboratory and biosafety practices. Cells were observed daily for visual Virus Cytopathic Effect CPE successively for 7 days.

Viral RNA Extraction and Real-time PCR 200 μ l harvest from virus culture flasks were collected periodically to perform RNA extraction by Viral DNA/RNA Extraction Kit-TIANLONG China followed by RT-PCR virus (Maccura EGN7103109 China). The kit was based on the multiplex PCR assay of ORF1ab, E and N gene of SARS-COV-2 virus. Real-time PCRs were performed at ABI 7500 (Applied Biosystems, USA).

Virus titration was performed through TCID₅₀ and plaque assay, end point assay in 96 well plates virus culture was utilized for this purpose. TCID₅₀/mL was quantified by using Spearman Kärber method. Moreover, a plaque assay for virus quantification was also conducted. Vero cells (120,000 cells) in complete growth medium DMEM were seeded in 24 well plates and incubated overnight. Next

day, cell growth medium was removed from the wells followed by 100 μ L inoculum of virus dilution ranging (10⁻³-10⁻⁷) in selected wells. Mock wells were added with serum reduced medium. The inoculum was removed followed by an hour of virus adsorption, and wells were washed two times with 200 μ L serum free medium and added with 700 μ L of plaque overlay medium (equal volume of 0.6% agarose and DMEM with 4% FBS) maintained melted at 56°C. The plate was incubated for 72 hours. Well content fixation was done with 400 μ L of 10% formaldehyde and kept at room temperature for an hour. The fixative along with agarose plugs was detached from the wells. Staining was done with 1% crystal violet in 20% ethanol for 15 minutes. Plaques at each dilution were counted and calculated as plaque forming unit per ml (pfu/mL).

Virus topography by Scanning Electron Microscopy virus culture supernatant was diluted in 1x PBS and filtration done with 0.2 μ M poly carbonate membrane (Merck Millipore). The trapped virus particles were primarily fixed with 2.5% glutaraldehyde, material rinsed with 1x PBS. The membrane was then placed with 1% Osmium Tetroxide and incubated at room temperature for 20 minutes. Membrane rinsed with 1X PBS. Sequential dehydration with increasing concentration (70%, 90% and 100%) of ethanol was carried out. The final drying was carried out in Hexamethyl Disilazane (HMDS). Membrane coating was performed on gold sputter and ready material was analyzed through Scanning Electron Microscope JSM-100 (Joel, USA).

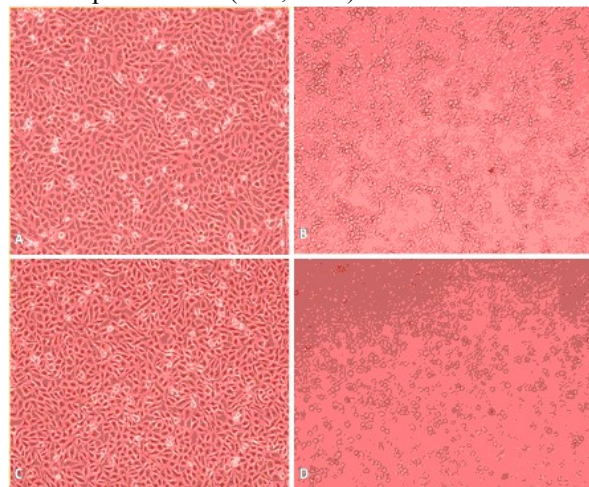


Fig. 1: A Mock vero CCL 81, B SARS COV 2 infected vero CCL 81 48hpi, C Mock vero E6 D SARS COV 2 infected vero E6

Virus Neutralization Assay Sera of two SARS-COV-2 convalescent patients were taken to perform virus neutralization test. Healthy (non SARS-COV-2) serum was served as negative control. All sera were treated for half hour incubation at 56°C for inactivation. An equal volume of virus stock was mixed with each dilution (1:2 to 1:2048) in separate tubes.



Fig. 2: A Mock Vero E6, B SARS-CoV-2 infected E6, C Viral infection in progress in presence of healthy control serum, D SARS-CoV-2 infection in presence of 1:16 diluted SARS-CoV-2 convalescent serum, E SARS-CoV-2 infection in presence of 1:32 diluted SARS-CoV-2 convalescent serum F SARS-CoV-2 infection in presence of 1:64 diluted SARS-CoV-2 convalescent serum, G SARS-CoV-2 infection in presence of 1:128 diluted SARS-CoV-2 convalescent serum

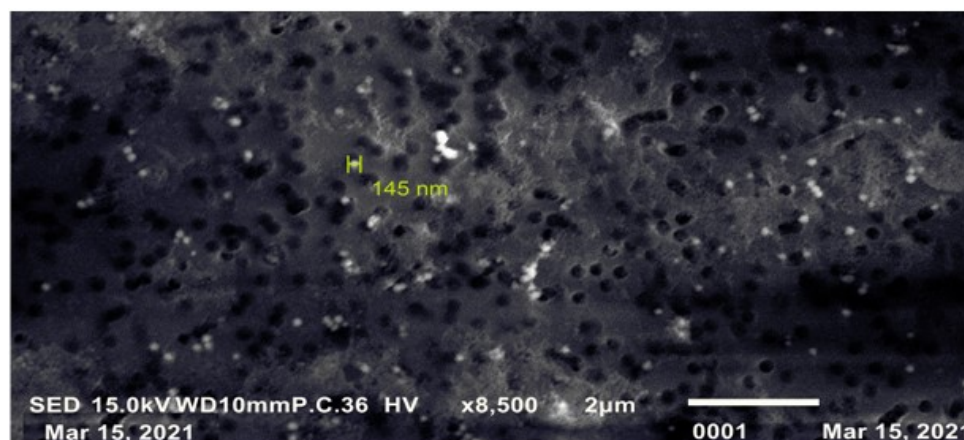


Fig. 3: Scanning electron micrograph of SARS-CoV-2 (Spherical particles in white color)

The virus serum mixture was incubated for an hour. Overnight-grown Vero cell (seeding density 20,000 cells/well) in 24 well plates was added with 100µL of serum and virus mixtures in each respective well. 100µL growth medium was added to cell control and 100µL diluted virus stock was added as virus control in respective well. Plate incubated at for virus adsorption. Plate washed thrice with serum free DMEM. Cells were incubated with serum reduced DMEM for 72hours. CPE observation was taken (Zhou *et al.*, 2020).



Fig. 4: *Sisybrium irio* seeds tested for *in vitro* antiviral activity against SARS-COV-2

Cytotoxicity Assay Vero cells (cell seeding density 15,000 cells/well) in DMEM growth medium (Gibco) were grown overnight. Serial two-fold drug dilutions were prepared starting from 0.5% to 0.000976% in growth medium and added to respective wells in triplicate along with no drug control (NDC) and blank. The plates were incubated for 72 hours. The cell viability was determined using MTT Kit (Sigma Aldrich, Cell Proliferation Kit I-Roche Cat. No.11 465 007-001). Well contents were aspirated and replenished with 10% MTT reagent prepared in growth medium. The plates were incubated for 4 hours at similar incubation condition. Solubilization buffer was added to each well plate incubated overnight in similar incubation conditions. The absorbance was measured at 570nm using Thermo Scientific Multiskan GO spectrophotometer.

Antiviral Assays Vero cells were grown overnight similarly as of cytotoxicity assay. Similar drug dilutions were prepared starting from 0.5% to 0.000488% in serum reduced DMEM and added with 100 TCID₅₀ SARS-CoV-2 to the respective wells in triplicate along with cells and virus controls. The two hour incubation was given for virus adsorption. The drug virus mixture was removed and wells were washed thrice with serum free DMEM. The treatment wells were replenished with respective drug dilutions. The plate was incubated for 72 hours. Any CPE inhibition was noted.

Table 1: CC50 and IC50 values of Khaksi extracts tested against SARS-COV-2 virus

Herb extract tested	CC50 (%)	IC50 (%)
Khaksi (Water extract)	0.1484	>1
Khaksi (DCM extract)	0.0979	>0.0078
Khaksi (Ethanol extract)	0.0466	>0.0039

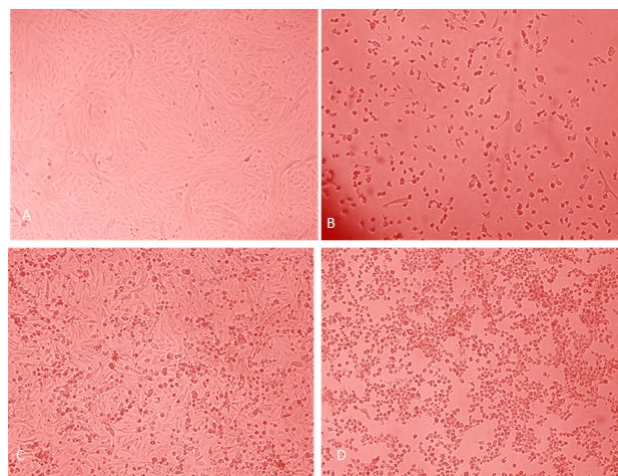


Fig. 5: A Mock Vero E6 confluent cells, B Vero E6 Cell cytotoxicity 24 hour post drug (*Sisybrium irio*) addition, C SARS-CoV-2 Moderate CPE 48hpi of Virus control and D SARS - CoV-2 Advanced CPE 72 hpi of virus control.

STATISTICAL ANALYSIS

Cell Cytotoxicity and Viral inhibition Analysis MTT absorbance readings were normalized to get % Cytotoxicity and % viral inhibition (Weston *et al.*, 2020) 50% Cell Cytotoxicity (CC₅₀) and 50% Viral Inhibitory Concentration (IC₅₀) were calculated and determined via using Graph Pad Prism Version 5.00.

RESULTS

Out of 10, 03 samples showed successful growth of SARS-CoV-2. Virus growth was preliminary confirmed by Real-time RTPCR. Later on only 01 SARS-CoV-2 virus was selected for further passages. We challenge the available cell lines Vero E6, Vero CCL 81, BHK and Huh 07 for rescuing the SARS-CoV-2 virus. It is observed that SARS-CoV-2 grown on Vero E6 and Vero CCL 81 was equally good while the virus did not show the cytopathic effect on BHK-21 and Huh 07. The isolated virus was sub cultured and propagated further three times on Vero CCL 81 cells. The fourth passage was stored and titrated as 10⁷ TCID₅₀/mL similarly pfu count was observed and calculated as 1.4 X 10⁶ pfu/mL).

In addition to Real time PCR, confirmation of the rescued virus was performed through Scanning Electron Microscopy (SEM) and virus neutralization assay. A

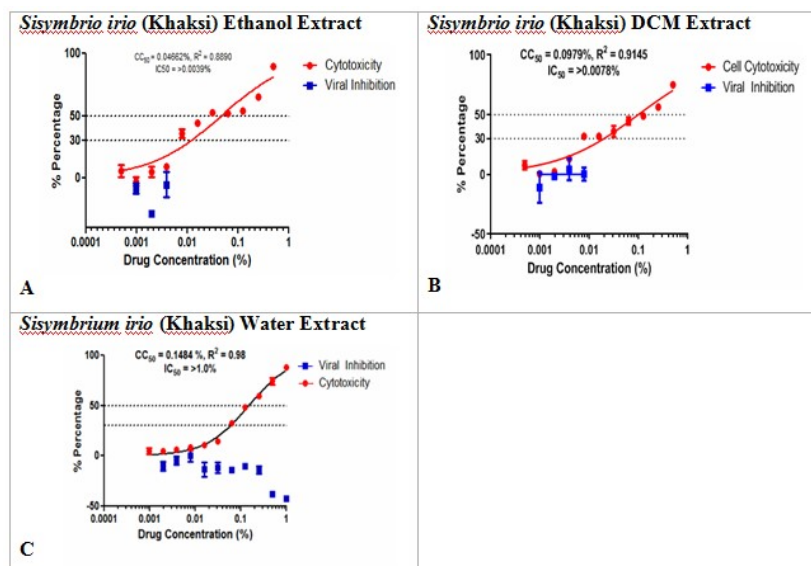


Fig. 6: Graphical Illustration of percentage cytotoxicity and percentage inhibition exhibited by A: *Sisymbrium irio* (Khaksi) ethanol extract, B: *Sisymbrium irio* (Khaksi) DCM extract and C: *Sisymbrium irio* (Khaksi) water extract.

prominent decline in Threshold Cycle (Ct) was observed in Real-time PCR in supernatants collected from Vero cells infected with patient nasopharyngeal processed samples. A precise reduction of 12-16 Ct was observed at 48 hour post-infection (48hpi) indicative of successful SARS-COV-2 rescue in susceptible cell line. The isolated virus was neutralized with SARS-COV-2 positive convalescent sera. Both sera were positively neutralized the SARS-COV-2 virus at the 100TCID₅₀/ml concentration as well as 1:16 and 1:64 serum dilutions showed grade 1 CPE and for 1:32 and 1:128 serum showed grade 4 CPE.

Sisymbrium irio (Khaksi) was tested for antiviral against SARS-COV-2 virus in the laboratory setting. All three extracts (water, ethanol and dichloromethane) were found to have cytotoxic (not safe) to the Vero E6 cell line. These provided cytotoxicity at very low concentration 0.1484%, 0.04662% and 0.0979% respectively. Viral inhibition at higher concentration did not make it possible to calculate a selective index of drug.

DISCUSSION

The reason for unsuccessful virus culture of samples in BHK-21 and Huh07 cell lines was investigated, and it is observed that samples were collected from different hospitals in different viral transport medium. This is suggestive of possible presence of virus growth inhibitor or may be the samples collected in lysis reagents containing transport medium, causative of cell line detachment during adsorption period which proves the right selection of viral transport medium plays an important role in performing virus culture. Successful SARS-COV-2 culture on Vero CCL 81 and Vero E6 were

in line with other reports (Ksiazek *et al.*, 2003; Peiris, *et al.*, 2003; Harcourt *et al.*, 2020; Araujo *et al.*, 2020; Sarkale *et al.*, 2020).

Sphere-shaped particles of approximately less than 150 nm through Scanning electron micrograph indicative of earlier reported morphology and size of SARS-CoV-2 (Zhu *et al.*, 2020; Laue *et al.*, 2021). Semi quantitative Virus neutralization with COVID specific convalescent sera and no neutralization effect with healthy control/ non SARS-COV-2 serum provides clear indication of successful virus rescue and neutralization.

Sisymbrium irio (Khaksi) did not found to be safe as exhibited cytotoxicity at a very low concentration of drug compare to the % viral inhibition. These results are in contrast to the broad-spectrum routine use of the Khaksi as an antipyretic and antibacterial ((Hailu *et al.*, 2019), though Khaksi was recommended as a co-therapy to treat COVID (Ansari *et al.*, 2020; Iqbal *et al.*, 2021). Furthermore, extracts of *Sisymbrium irio* which were reported to have antibacterial effect (Vohora, *et al.*, 1980; Javaid *et al.*, 2017) could not be established on *in vitro* cell lines. It may because the antibacterial and antifungal activities were tested on bacteria and fungus is not involved with the live cell testing. Secondly it leads to be precise *in vitro* cell line selection which is near human model like human lung carcinoma cell line.

Virus isolation was performed as it is the very crucial and fundamental step to carrying out *in vitro* research and development purpose like therapeutic, environmental and virus genome sequencing studies. More elaborated virus culture studies are required on other type of cell lines, like, lung epithelial cell lines and other human cell lines.

Moreover, lung epithelial cell lines may be defined as better model to conduct antiviral studies.

CONCLUSION

Virus culture is a basic fundamental step for in vitro therapeutic studies and drug development studies. Appropriate cell line selection for the virus growth and drug testing is the key factor. Khaksi is a known antipyretic, antifungal and ant parasitic agent, but could not be found effective against SARS COV 2. Further in vitro drug testing against SARS COV 2 and other respiratory viruses may be performed on other cell lines to define an appropriate cell line model.

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REFERENCES

- Ansari AP, Ahmed NZ, Ahmed KK and Khan AA (2020). An insight on Wabāi Amrād (epidemic diseases) and COVID-19 like conditions-Unani perspective. *IJCRR*, **12**(17): 109-119.
- Araujo DB, Machado RRG, Amgarten DE, Malta FDM, de Araujo GG, Monteiro CO, Candido ED, Soares CP, de Menezes FG, Pires ACC and Santana RAF (2020). SARS-CoV-2 isolation from the first reported patients in Brazil and establishment of a coordinated task network. *Memórias do Instituto Oswaldo Cruz*, **115**: e200342.
- Hailu T, Gupta RK and Rani A (2019). *Sisymbrium irio* L: A herb used in the Unani system of medicine for broad spectrum therapeutical applications. *IJTK*, **18**(1): 140-143.
- Harcourt J, Tamin A, Lu X, Kamili S, Sakthivel SK, Murray J, Queen K, Tao Y, Paden CR, Zhang J and Li Y (2020). Severe acute respiratory syndrome coronavirus 2 from patient with coronavirus disease, United States. *Emerg. Infect. Dis*, **26**(6): 1266-1273.
- Iqbal A, Rashid A, Quraishi HA, Jan A and Ahmad SZ, (2021). Corona virus and traditional approach for developing immunity through Unani herbal medicine. *J. Pharamacovigil.*, **9**(1): 1-8.
- Javaid A, Afzal L and Shoaib A (2017). Antifungal potential of a brassicaceous weed *Sisymbrium irio* against *Macrophomina phaseolina*. *Planta Daninha*, **35**: e017164280.
- Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W and Rollin PE (2003). A novel coronavirus associated with severe acute respiratory syndrome. *NEJM*, **348**(20): 1953-1966.
- Laue M, Kauter A, Hoffmann T, Moller L, Michel J and Nitsche A (2021). Morphometry of SARS-CoV and SARS-CoV-2 particles in ultrathin plastic sections of infected vero cell cultures. *Sci. Rep.*, **11**(1): 1-11.
- Mickymaray S (2019). One-step synthesis of silver nanoparticles using Saudi Arabian desert seasonal plant *Sisymbrium irio* and antibacterial activity against multidrug-resistant bacterial strains. *Biomolecules*, **9**(11): 662.
- Peiris JSM, Lai ST, Poon LLM, Guan Y, Yam LYC, Lim W, Nicholls J, Yee WKS, Yan WW, Cheung MT and Cheng VCC (2003). Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet*, **361**(9366): 1319-1325.
- Saqib M, Sieberg A, Hussain MH, Mansoor MK, Zohaib A, Lattwein E, Muller MA, Drosten C and Corman VM (2017). Serologic evidence for MERS-CoV infection in dromedary camels, Punjab, Pakistan, 2012-2015. *Emerg. Infect. Dis*, **23**(3): 550.
- Sarkale P, Patil S, Yadav PD, Nyayanit DA, Sapkal G, Baradkar S, Lakra R, Shete-Aich A, Prasad S, Basu A and Dar L (2020). First isolation of SARS-CoV-2 from clinical samples in India. *IJMR*, **151**(2-3): 244.
- Vohora SB, Naqvi SAH and Kumar I (1980). Antipyretic, analgesic and antimicrobial studies on *Sisymbrium irio*. *Planta Med*, **38**(03): 255-259.
- Weston S, Coleman CM, Haupt R, Logue J, Matthews K, Li Y, Reyes HM, Weiss SR and Frieman MB (2020). Broad anti-coronavirus activity of food and drug administration-approved drugs against SARS-CoV-2 in vitro and SARS-CoV *in vivo*. *JVI*, **94**(21): e01218-20.
- Zheng J, Hassan S, Alagaili AN, Alshukairi AN, Amor NM, Mukhtar N, Nazeer IM, Tahir Z, Akhter N, Perlman S and Yaqub T (2019). Middle East respiratory syndrome coronavirus seropositivity in camel handlers and their families, Pakistan. *Emerg. Infect. Dis*, **25**(12): 2307-2309.
- Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL and Chen HD (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*, **579**(7798): 270-273.
- Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R and Niu P (2020). A novel coronavirus from patients with pneumonia in China, 2019. *NEJM*, **382**: 727-733.
- Zohaib A, Saqib M, Athar MA, Chen J, Sial AUR, Khan S, Taj Z, Sadia H, Tahir U, Tayyab MH and Qureshi MA (2018). Countrywide survey for MERS-coronavirus antibodies in dromedaries and humans in Pakistan. *Virol Sin.*, **33**(5): 410-417.