Cent. Eur. J. Biol. • 9(5) • 2014 • 531-542
DOI: 10.2478/s11535-013-0276-8

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Central European Journal of **Biology**

Exploration of the *in vitro* cytotoxic and antiviral activities of different medicinal plants against infectious bursal disease (IBD) virus

Research Article

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Received 11 September 2013; Accepted 28 October 2013

Abstract: Infectious bursal disease (IBD) caused by non-enveloped double stranded RNA virus is an acute and contagious poultry disease. Outbreak of IBD could result in 10-75% mortality of the birds; hence it has gained socio-economic importance worldwide. Medicinal plants have shown broad spectrum anti-viral activities against RNA and DNA viruses. Moringa oleifera Lam (MOL), Phyllanthus emblicus Linn (PEL), Glycyrrhiza glabra Linn (GGL), and Eugenia jambolana Lam (EJL) are commonly available medicinal plants of the sub-continent and exhibited anti-viral potential against different viruses. Ethanolic extracts of the leaves of MOL and EJL, roots of GGL and dried fruit of PEL were investigated for their cytotoxic and anti-viral potential against IBD virus using MTT colorimetric assay and anti-viral assay. Significant anti-viral potential ($P<0.001$) was demonstrated at concentrations 12.5, 25, 50 and 100 μg ml^{-1} of GGL, PEL, MOL and EJL, respectively, with no cytotoxicity. Data also spotlighted that all tested plant extracts possess significant anti-viral potential and this trend was higher in GGL followed by PEL, MOL, and EJL. The data undoubtedly conclude that these medicinal plants contain several health beneficial phyto-chemicals which got significant anti-viral potential and effectively be utilized against IBD virus. Moreover, the outcomes of this study provide a platform on the way to discover novel anti-viral agents against IBD virus and other viruses from plant origin.

Keywords: *IBD • Medicinal plants • Cytotoxicity • Anti-viral*

1. Introduction

Infectious bursal disease (IBD) is caused by virus from Birnaviridae family of genus Avibirna virus. IBD is an acute and contagious disease also known as Gumboro disease and has gained socio-economic importance at the international level [1]. IBD affects young chicks from 3-6 weeks of age [2] and based on the virulence IBD has been categorized into three categories *i.e.*, a) mild b) virulent and c) very virulent. Virulent strain was first identified in early 1960s in United States and produced hemorrhagic lesions with depletion of B cells with mortality rate of 30-60% in size. "Very virulent" strain appeared in several European and Asian countries during mid 1990s with more than 70% mortality in chicks. The clinical signs of which were comparable to virulent strain [3].

IBD virus is a double stranded RNA virus, which is non-enveloped and hard in nature. The capsid of the IBD virion consists of several structural proteins, which has ability to survive in poultry houses for several years despite extensive cleaning and disinfection. IBD virus replicates in the bursa of fabricius and after completion of the pathogenesis, B cells deplete in bursal of fabricus resulting atrophy of that region. Several viral strains of IBD have been identified in bursa, spleen and cecal tonsils [4] and both humoral and cell based immunities are compromised during this disease [5]. There are two mechanisms by which T cells modulate IBD virus pathogenesis. Firstly, viral replication is reduced in bursa at the preliminary stage (at five days of infection) of disease by T cells. Secondly they enhanced bursal tissue damage and delay tissue recovery by their

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cytotoxic effects by releasing cytokines [6]. Thus Gumboro disease causes immunosuppression that results in increased mortality and significant economic losses to the poultry industry annually.

Currently, there is no effective treatment for the Gumboro infected birds and preventive vaccination is the only remedy adopted in the poultry industry. Hence, there is a dire need to investigate different indigenous medicinal plants with anti-viral properties. The use of medicinal plants as therapeutic agent has long history since the existence of human civilization [7]. Industrial revolutions has changed the research trends to synthesize the chemicals as medicine rather to evaluate the natural plants for their medicinal activity, however 25% drugs used worldwide come from plant origin. The world health organization (WHO) has been promoting the use of phyto-chemicals for therapeutical uses in the developing countries like Pakistan [8].

Medicinal plants have shown broad spectrum anti-viral activities against RNA and DNA viruses [9,10], which could contribute as a promising candidate in controlling the life threatening viral infections [11]. Increased incidences of viral resistances and emergence of new viral diseases have led the scientists to explore for new anti-viral compounds [12,13]. Thus more and more research in medicinal plants is the demand of modern age. Based on these criteria, we have used extracts of four well known plants *i.e.*, Moringa oleifera Lam (MOL), commonly known as Sonajna (Figure 1A), Phyllanthus emblicus Linn (PEL), traditionally known as Aamla (Figure 2A), Glycyrrhiza glabra Linn licorice (GGL), commonly known as Malathi (Figure 3A), and Eugenia jambolana Lam (EJL), traditionally known as Jammun, (Figure 4A) to investigate their cytotoxic and anti-viral potential against IBD virus.

MOL has been traditionally used as diuretic agent, anti-inflammatory agent, anti-spasmodic agent, anti-tumor agent [14], to support immune system, to inhibit gastric lesions [15], to improve vision, in mental alertness and osteoporosis [16]. Moreover, MOL has also been described to have inhibitory activity against Herpes simplex virus type 1 [17].

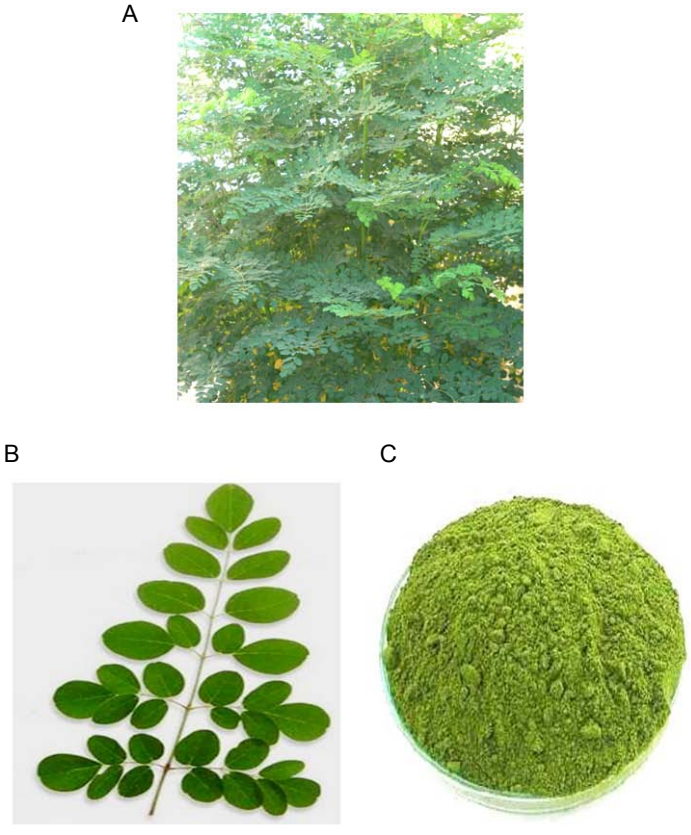


Figure 1. Pictorial presentation of Moringa oleifera tree (A), leaves (B) and crude powder (C) used to obtain extract for the study. a) http://jessaskin.com/wp-content/uploads/2011/03/682moringa.jpg b) http://moringaextract.net/wp-content/uploads/2012/10/moringa-leaf-superfood.jpg

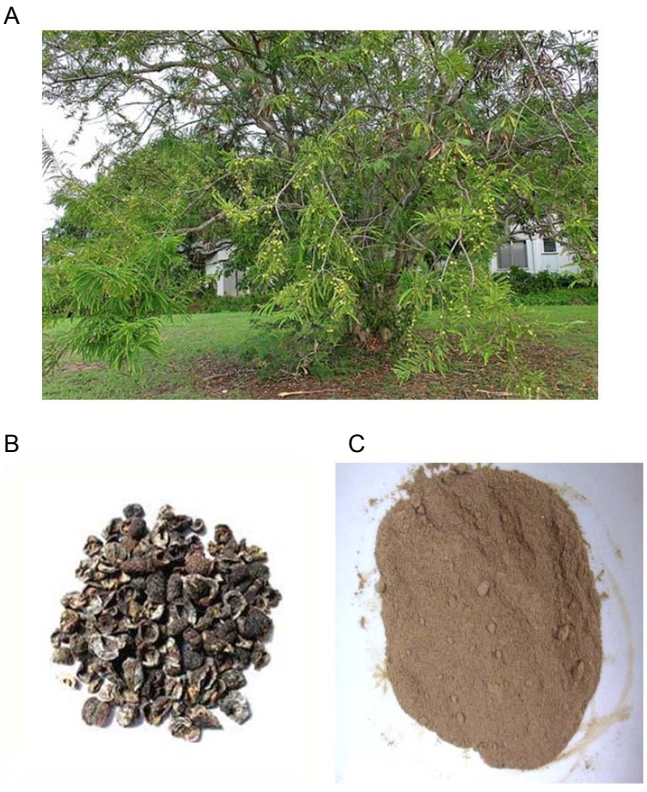


Figure 2. Pictorial presentation of Phyllanthus emblicus tree (A), dried fruit (B) and crude powder (C), used to determine cytotoxic and anti-viral potential of the plant. a) http://gcdist.com/seedstore/index.php?page=detail&get_id=95&category=11

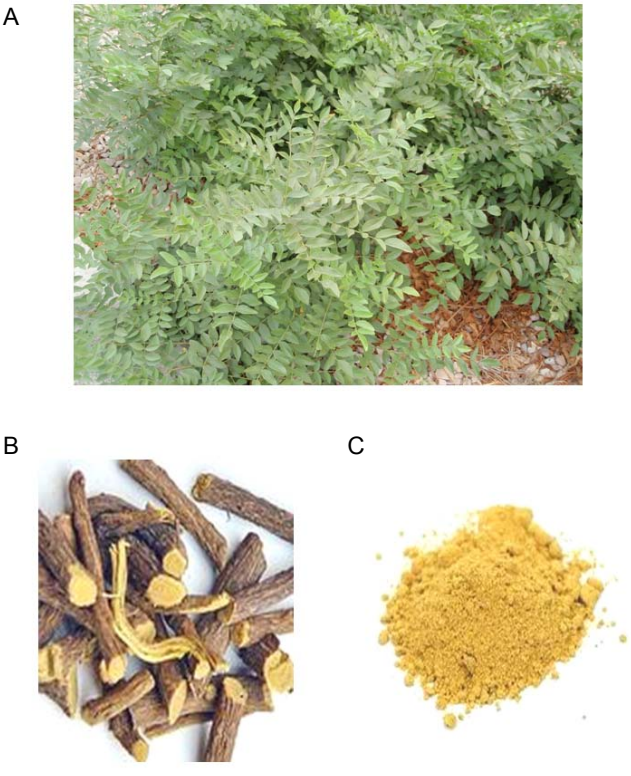


Figure 3. Pictorial presentation of Glycyrrhiza glabra plant (A), dried roots (B) and crude powder (C) used to obtain extract for the study a)http://www.spicesmedicinalherbs.com/img/glycyrrhiza-glabra.jpg

A

B

Figure 4. Pictorial presentation of Eugenia jambolana tree (A) and crude powder (B) used to determine cytotoxic and anti-viral potential of the plant a) http://www.imra-ratsimamanga.org/ej.jpg

On the other hand, PEL is traditionally used to control hepatitis, regulation of stomach movements, cancer, and act as immunomodulator [18]. Likewise, non sesquiterpenoids obtained from PEL has shown anti-viral activity against Coxsachie virus B_3 [19]. GGL has traditionally been used as anti-inflammatory agent, anti-bacterial agent, expectorant, mild laxative and diuretic [20]. The extract from GGL has also been used to treat Kaposi sarcoma-associated herpes virus (KSHV) [21], influenza virus [22], HIV-1 [23], vaccinia virus, ND virus, vesicular stomatitis virus [24], respiratory syncytial virus [25], and herpes viruses [26]. Similarly, EJL is traditionally used as anti-bacterial, anti-inflammatory, anti-ulcerogenic, cardio-protective, anti-allergic, anti-cancer, anti-oxidant, hepatoprotective, anti-diarrheal and hypoglycemic [27]. The leave extract of EJL inhibits goat-pox virus replication *in vitro* and all above mentioned plants has shown significant activities against RNA and DNA viruses [28]. Hence, this project was designed to ascertain cytotoxic and anti-viral potential of extracts from MOL, PEL, GGL and EJL plants against IBD virus using MTT colorimetric assay and anti-viral assay.

2. Experimental Procedures

2.1 Extraction

The leaves of MOL and EJL were collected from the University of Veterinary and Animal Sciences Lahore, while dried fruits of PEL and roots of GGL were purchased from the local market. All collected plant materials were identified by a botanist from the Department of Botany, Government College University, Lahore, Pakistan. Collected plant materials were washed with distilled water and shade dried. The materials were then grinded to a fine powder before extraction. 100 grams of the dried powder were introduced to thimble of Soxhlet (CG-1368 China) and ethanol extraction (500 ml) was carried out for 24 hours at 40°C [29]. The extracts were centrifuged (2400 × *g*) for 15 minutes and were stored at -20^{0}C until further use [30]. The extracts were then filtered using autoclaved filter papers (Whatmann No. 1, 0.45 µm pore size) in a safety cabinet and dried in an oven at 45°C. Finally two fold dilutions *i.e.*, 100, 50, 25 and 12.5 µg ml^{-1} were prepared from dried extracts in 1% DMSO solution [31].

2.2 Virus Stock

Purified and well characterized IBD virus was obtained from quality operation laboratory (QOL), University of Veterinary & Animal Sciences, Lahore, Pakistan. Tissue culture infective dose ($TCID_{50}$) was determined for the virus adopting the method described by the Reed and Munch [32].

2.3 Cell Line

Vero cell line was obtained from QOL, University of Veterinary and Animal Sciences, Lahore, Pakistan and viability of the cells was determined by using "dye exclusion method", as described by Freshney and Raheel [33,34]. Moreover, following formula was used to determine the % viable cells.

$$\% \text{ Viable cells} = \frac{\text{Number of viable cells / ml}}{\text{Total number of cells / ml}} \times 100$$

2.4 Seeding of Vero cells in 96-well cell culture plates

For both cytotoxicity and anti-viral assay, 100 µl of 1 × 10^5 ml^{-1} of the cell suspension, prepared in M199 media containing 10% fetal bovine serum, were seeded in each well of 96-well plates. The plates were then incubated at 37^{0}C for 72 hrs with 5% CO_2. Cells in

each plate were regularly monitored under an inverted microscope (Olympus CK40, Japan) until they reached to a confluency of 80 – 90% [33,34].

2.5 Cytotoxicity assay

Exactly identical method of cell culture and washing was adopted as reported by Freshney *et al.* and Raheel *et al.* [33,34]. Briefly, 100 µl of the test materials were added to 80-90% confluent cells grown in freshly prepared M199 media, while Vero cells grown in M199 served as positive control, while DMSO (20%) and M199 media was used as negative control [33,34].

2.6 Anti-viral assay

Exactly identical method of cell culture and washing was adopted as reported by Raheel *et al.* and Birch [34,35]. Briefly, plant extracts were mixed with IBD virus (10^6 $TCID_{50}$) and 100 µl were introduced to Vero cell containing wells.

2.7 MTT assay to quantify viable cells

Exactly identical method of cell culture and washing was adopted as reported by Raheel *et al.* and Ejaz *et al.* [34,35]. Briefly, cells were incubated with 100 µl of 0.5% MTT solution, then 100 µl of DMSO (5%) was added and finally optical density (OD) was determined by ELISA [34,36].

Following formula was used to calculate cell survival percentage (CSP) in both cytotoxic and anti-viral assay.

$$CSP = \frac{\text{Mean OD of test - Mean OD of negative control}}{\text{Mean OD of positive control}} \times 100$$

2.8 Statistical analysis

The data was analyzed by statistical package for social sciences (SPSS for Windows version 12, SPSS inc., Chicago, IL, USA). The results were evaluated as CSP and expressed in terms of means ± S.D. Analysis of variance (ANOVA) and Post hoc t-test was applied to analyze data and $P < 0.05$ was considered as significant [34,37].

3. Results

3.1 MTT Assay

For each extract, cytotoxic activity was determined by calculating cell survival percentage at concentrations 100, 50, 25 and 12.5 µg ml^{-1}. In MOL treated groups, the cell survival percentage was 84.48%, 90.32%, 77% and 81.90% with no statistical significance with respect to the control group (Figure 5A) respectively, while it was 1%, 75%, 87.4% and 88%, respectively for the PEL extract (Figure 5B). The data spotlight that all the tested concentrations of MOL were non cytotoxic to Vero cell line (Table 1, Figure 5A), while in PEL treated groups the concentration 100 µg ml^{-1} proved cytotoxic ($P<0.001$) to Vero cell line (Table 1, Figure 5B).

Cytotoxic analysis of GGL elaborated that concentrations 100 and 50 µg ml^{-1} were cytotoxic to Vero cells and cell survival was 1% ($P<0.001$) and 29% ($P<0.05$), respectively (Figure 6A). However, cell survival of 84.5% and 90% was observed at concentrations 25 and 12.5 µg ml^{-1}, respectively, which enlighten that these concentrations are safe for Vero cell line. In case of EJL treated groups, the cell survival percentage was 82.8%, 76%, 77% and 78% at concentrations 100, 50, 25, and 12.5 µg ml^{-1}, respectively, spotlighting that all tested concentrations are non-cytotoxic for Vero cell line with no statistical significance (Table 1, Figure 6B).

3.2 Anti-viral assay

In anti-viral assay, MOL treated groups demonstrated cell survival percentage of 80.03%, 75%, 15% and 4.48%, respectively. The result showed that 100 and 50 µg ml^{-1} got considerable anti-viral potential ($P<0.001$) for IBD virus (Figure 5A). Anti-viral analysis of PEL demonstrated that 50 and 25 µg ml^{-1} has highly significant ($P<0.001$) cell survival percentage of 81.03% and 72%, respectively. However, at concentrations 100 and 12.5 µg ml^{-1}, the cell survival percentage was 11% and 9%, respectively (Table 2, Figure 5B).

No.	Conc. (µg ml^{-1})	Mean cell survival percentage ± Standard deviation			
		Moringa oleifera (n=3)	Phyllanthus emblicus (n=3)	Glycyrrhiza glabra (n=3)	Eugenia jambolana (n=3)
01	100	84.48±1.79[bc]	1±0.2[ad]	1±0.21[ad]	82.8±0.461[bc]
02	50	90.32±4.44[c]	75±3.78[c]	29±2.1[abd]	76±0.33[c]
03	25	77±1.05	87.4±1.9	84.5±0.87	77±0.446
04	12.5	81.90±0.309	88±1.86	90±1.57	78±0.587

Table 1. Cytotoxic potential of the ethanolic extract of Moringa oleifera, Phyllanthus emblicus, Glycyrrhiza glabra and Eugenia jambolana.

[a] *significant difference from Moringa oleifera at the 0.05 level*
[b] *significant difference from Phyllanthus emblicus at the 0.05 level*
[c] *significant difference from Glycyrrhiza glabra at the 0.05 level*
[d] *significant difference from Eugenia jambolana at the 0.05 level*

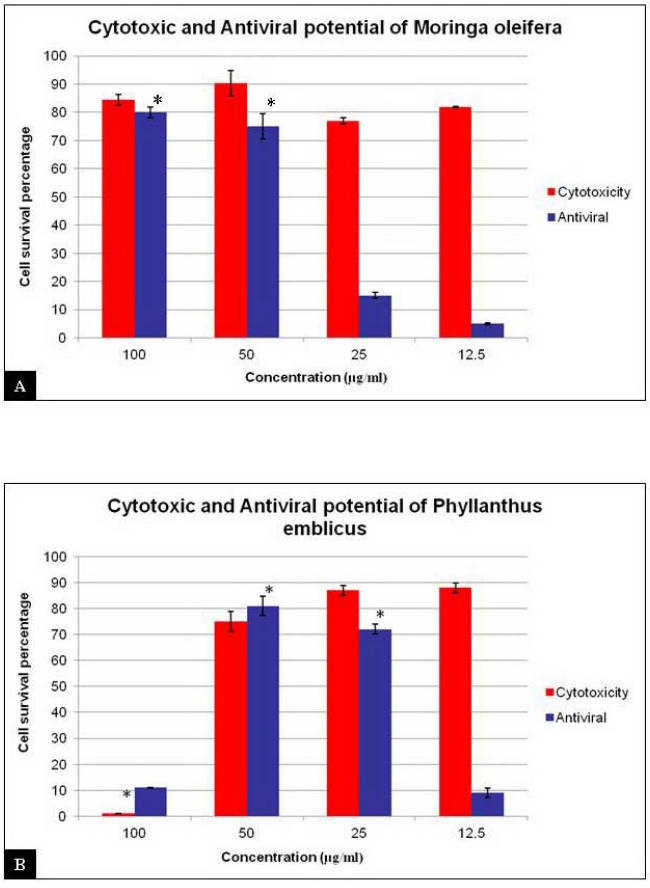


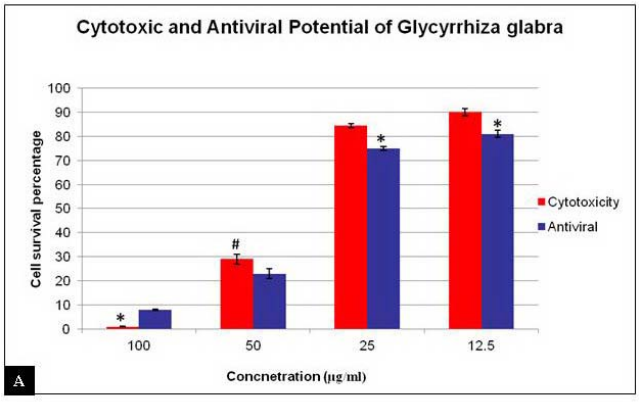
Figure 5. Graphical representation of the cytotoxic and anti-viral potential of Moringa oleifera (A) and Phyllanthus emblicus (B). * Significant increase ($P<0.001$)

In GGL treated groups, the cell survival percentage was 8%, 23%, 75% and 81%, respectively (Figure 6A). It is evident from the data that concentrations 25 and 12.5 µg ml^{-1}, which were not cytotoxic, exhibited prominent anti-viral activities ($P<0.001$) against IBD virus with cell survival percentage of 75% and 81%, respectively. In EJL treated groups, the cell survival percentage of 62%, 38%, 7.53% and 12.4%, respectively, was observed for the said concentrations against IBD virus. It was evident from the data that concentration of 100 µg ml^{-1} has highly significant anti-viral property ($P<0.001$) with cell survival percentage of 62% and concentration 50 µg ml^{-1} has significant anti-viral activity ($P<0.05$) with cell survival percentage of 38% (Table 2, Figure 6B).

4. Discussion

Poultry industry all over the world put emphasis on IBD [38] because outbreaks of IBD could result in huge losses ranging from 10-75%, 20-30% mortality in broiler [39,40] and 40-80% mortality in layer chicks [41]. IBD adversely affects chicken immune system and described as AIDS of the chicken. It invades bursa fabricus, an organ responsible for producing antibodies in chicken and completely destroyed the organ, resulting higher (36.65-40.40%) mortality in egg type layers [42-44]. Birds of all ages are vulnerable to IBD but fatalities (20-76%) are observed between the age of 2-12 weeks [45]. Egg type layers are more susceptible to IBD because of poor vaccination [46,47], filthy environment and factors like concomitant infections with *E. coli*, coccidiosis and other bacterial infections [48].

It is believed that vaccination against IBD at the age of 14-21 days partially control the problem, explaining that despite the vaccination, atrophy of bursa could not be protected even if there is a mild infection of IBD [49]. It is difficult to develop new anti-viral agents because of poor selective toxicity and development of resistance against the existing drugs [50]. It has been documented by the researchers that extracts of medicinal plants



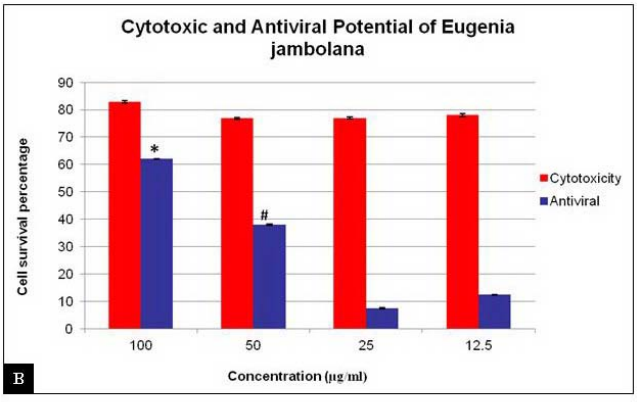


Figure 6. Graphical representation of cytotoxic and antiviral potential of Glycyrrhiza glabra (A) and Eugenia jambolana (B). *Significant increase ($P<0.001$) #Significant increase ($P<0.05$)

No.	Conc. ($\mu g\ ml^{-1}$)	Mean cell survival percentage ± Standard deviation			
		Moringa oleifera (n=3)	Phyllanthus emblicus (n=3)	Glycyrrhiza glabra (n=3)	Eugenia jambolana (n=3)
01	100	80.03 ± 1.35^{bc}	11 ± 0.35^{ad}	8 ± 0.28^{ad}	62 ± 0.152^{bc}
02	50	75 ± 4.3^{cd}	81.03 ± 3.94^{cd}	23 ± 1.98^{ab}	38 ± 0.208^{ab}
03	25	15 ± 0.98^{bcd}	72 ± 2.1^{ad}	75 ± 0.93^{ad}	7.53 ± 0.187^{bc}
04	12.5	4.48 ± 0.57^{c}	9 ± 1.64^{c}	81 ± 1.48^{abd}	12.4 ± 0.089^{b}

Table 2. Anti-viral potential of the ethanolic extract of Moringa oleifera, Phyllanthus emblicus, Glycyrrhiza glabra and Eugenia jambolana.

[a] significant difference from Moringa oleifera at the 0.05 level
[b] significant difference from Phyllanthus emblicus at the 0.05 level
[c] significant difference from Glycyrrhiza glabra at the 0.05 level
[d] significant difference from Eugenia jambolana at the 0.05 level

possess certain phyto-chemicals that can cure ailments. Much interest has been shown by scientists around the globe for the prevention and treatment of viral diseases by medicinal plants because existing anti-viral drugs have much adverse effects and have shown to develop resistance [51]. The screening of plants as a possible source of anti-viral has led to the discovery of several medicinal plants, the extracts of which are effective against several viruses *e.g.*, Herpes simplex virus [11,52,53].

Increased mortality and economic losses associated with IBD emphasize to discover potent anti-viral agents against IBD virus from natural sources. For this, we designed current project to appraise anti-viral potential

of MOL, PEL, GGL and EJL against IBD virus. The cytotoxic and anti-viral potential was determined at concentrations 100, 50, 25 and 12.5 µg ml^{-1}. In case of groups treated with the extract of MOL, all the above described concentrations were safe for the Vero cell line with cell survival percentage of 84.48%, 90.32%, 77% and 81.90%, respectively; with no statistical difference from the control group. These results are in agreement with the study of Saetung et al in which ethanolic and water extracts of MOL showed cytotoxicity at concentration above 100 µg ml^{-1} [54]. In another study, *in vitro* cytotoxic potential of MOL leaves using different solvents including ethanol was evaluated using "Neutral red dye up take assay" against different human myeloma cell lines and was found to exhibit a concentration dependent dose response [55]. Furthermore, extract from the leaves of MOL significantly reduced OH^- produced damage of pUC18 plasmid DNA [56] and also has anti-oxidant activity [57].

The extract from MOL at concentrations 100 and 50 µg ml^{-1} exhibited anti-viral potential ($P<0.001$) with cell survival percentage of 80.03% and 75%, respectively. Alcoholic extract of MOL bark and leaves has been reported to work against equine herpes virus type1 10^1 10^2 $TCID_{50}$ ml^{-1} of virus titer [58]. Aqueous methanloic seed extract of MOL is documented to have anti-viral potential against Herpes Simplex virus type 1 (HSV-1) and Polio virus type 1 [59]. Extract of MOL is also reported to enhance mean survival time, reduce the mortality of HSV-1 infected mice and delayed the development of skin lesions [17]. Moreover, MOL has been demonstrated to have anti-oxidant activity by scavenging peroxyl and superoxyl radicals [60,61], anti-bacterial activity [62,63] and anti-fungal activities [64].

The findings elaborated that fruit extract from PEL was cytotoxic at 100 µg ml^{-1} to the Vero cell line and exhibited only 1% cell survival ($P<0.001$) at the said concentration. However, all other concentrations appeared safe for the Vero cell line, which is in agreement to the statement by Ngamkitidechakul et al that PEL possesses cytotoxic activity against different cancer cell line but not cytotoxic against MRC5 (normal lung fibroblast) cell line [65]. In anti-viral assay, the extract from PEL demonstrated anti-viral activities against IBD virus at concentrations 50 and 25 µg ml^{-1} with cell survival percentage of 81.03% and 72% ($P<0.001$), respectively. The results are in accordance with the findings of El-Mekkawy, who demonstrated significant inhibitory effects of PEL extract on HIV-1 reverse transcriptase [66]. The fruit of PEL alone or in combination with *Punica granatum* has recently been used to treat jaundice and hepatitis [67]. It has been established that Ellagitannin isolated from PEL inhibited coxsackie virus B3, a major contributor of viral myocarditis, with IC_{50} value of 7.75 ± 0.15 µg ml^{-1} against HeLa cell line [68]. Moreover, PEL extract is also effective against herpes simplex virus (HSV-1) [69], and exhibited anti-viral activity against hepatitis B virus cultured on hepatoma cell line $HepA_2$ [70].

The extract of GGL exhibited anti-viral activity ($P<0.001$) at concentrations 25 and 12.5 µg ml^{-1} with cell survival percentage of 75% and 81%, respectively and both tested concentrations were non cytotoxic. Glycyrrhizin, a triterpenoid glycoside and glycyrrihizic acid isolated from GGL roots has been reported to have anti-viral activities against encephalitis virus and herpes simplex virus, respectively [24,71]. Alcoholic extract from the roots of GGL also possessed different health beneficial effects including anti-microbial activities [72,73], anti-oxidant activity [74], anti-angiogenic activities [75] and also actively worked against Mycobacterium tuberculosis [76].

All tested concentrations in EJL extract appeared safe with no significant cytotoxicity, while anti-viral activity was only observed at 100 µg ml^{-1} ($P<0.001$) with cell survival percentage of 62%. Our results are in agreement with Bhanuprakash *et al.*, who demonstrated that leave extract of EJL got significant anti-viral potential against goat-pox virus. Further he stated that 1999±0.5 mg ml^{-1} concentration of EJL extract in plaque reduction assay produced 99.92% inhibition of goat-pox virus [28] and 98.52% inhibition of buffalo-pox virus with non-toxic effects [77]. Other health beneficial effects of ethanolic extract of dried EJL seeds include anti-diabetic activity and gastric healing potential [78]. Leave extract from EJL has potential to scavenge nitric oxide (NO) and can be used to treat conditions with disproportionate production of NO [79]. Hydro-alcoholic extract of leaves from EJL is considered effective against multi-resistant strains of Pseudomonas aeruginosa, Klebsiella pneumonia, *Staphylococcus aureus* and *Candida krusei* [80].

5. Conclusions

It is evident from the study that all four tested plants contain significant anti-viral potential and the trend is higher in GGL 12.5 µg ml^{-1} ($P<0.001$), followed by PEL 25 µg ml^{-1} ($P<0.001$), MOL 50 µg ml^{-1} ($P<0.001$) and EJL 100 µg ml^{-1} ($P<0.001$). The data undoubtedly conclude that these medicinal plants contain several health beneficial phyto-chemicals which got significant anti-viral potential. Hence, the extract from these plants

could be used to improve the health status in certain pathological conditions but can also be effectively utilized against IBD virus. Further characterization of each extract is unavoidable to determine active anti-viral agents in these extracts and is currently ongoing in our laboratory.

Acknowledgements

The authors acknowledge financial support for this research from M.Phil evening program of the Department of Pharmacology and Toxicology, University of Veterinary and Animal Sciences Lahore, Pakistan.

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